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ON THE COVER: Schools of Medical Laboratory Science (MLS) are an essential resource to supply clinical laboratories with qualified technical personnel. Over the past few decades, many MLS programs in the US fell victim to shrinking budgets, foreshadowing the shortage of graduates that remains today. The COVID-19 pandemic created additional challenges, such as remote learning. However, a considerable portion of MLS training requires hands-on laboratory experience, so laboratory personnel were very much among the essential health care personnel during the pandemic. In this issue of *Laboratory Medicine*, Duzan and colleagues report the results of their survey of MLS graduates of nationally accredited training programs to assess the impact of the pandemic on their training and preparedness for the Board of Certification examination. Also in this issue, Smith et al, discuss the results of a survey to assess attitudes toward research among MLS professionals.

Statistical recommendations for ASCP journals

Michal Ordak, PhD

The American Society for Clinical Pathology (ASCP) provides an extensive collection of books covering a diverse range of subjects pertinent to the fields of pathology and laboratory medicine. Additionally, ASCP publishes three esteemed journals: *American Journal of Clinical Pathology* (AJCP), *Laboratory Medicine*, and *Critical Values*, which are leading sources of scientific and society information in print and online. The ASCP brings together over 100,000 anatomic and clinical pathologists, residents, fellows, medical laboratory professionals, and students with the shared goal of advancing laboratory medicine to enhance patient care through knowledge, collaboration, and global community. Biostatistics play a crucial role in laboratory medicine, as statistical analysis is fundamental to experiment design, data analysis, monitoring and quality control of laboratory processes, health risk assessment, and modeling and forecasting.[1,2] Unfortunately, the quality of statistical reporting is highly variable, and misapplied statistical analyses can contribute to the spread of misinformation and diminish trust in science.[3,4] Therefore, greater emphasis should be placed on adhering to statistical recommendations in biomedical journals. As the biostatistics editor of *Laboratory Medicine*, I recommend implementing the following: first, the ASCP journals websites should include basic statistical recommendations that authors should familiarize themselves with before submitting their manuscript. One of the recommended options is the SAMPL (Statistical Analyses and Methods in Published Literature) guidelines, aimed at improving the quality of statistical reporting.[5,6] The second recommendation is that common statistical errors made by authors, and the proper ways to correct them, should be the subject of presentations at the ASCP annual meetings. *Laboratory Medicine* also publishes various podcasts, and the third recommendation is to record podcasts focused on statistical methods and feature them on ASCP journal websites. These podcasts should cover various statistical topics, such as how to conduct a meta-analysis, measures of effect size, parametric vs nonparametric equivalents of statistical tests of significance, etc. A final recommendation is for more rigorous review of statistical analyses reported by authors in manuscripts submitted to ASCP journals. Authors should be encouraged to confirm, in their cover letter, their familiarity with the statistical recommendations provided by the journal and to identify who was responsible for the statistical analysis. Whenever doubts arise about the quality of the statistical analysis reported in a manuscript, a statistical review is highly recommended. Implementing these recommendations will help improve the quality of statistical reporting.

SPECIAL REPORT

Impact of COVID-19 pandemic on accredited programs and graduates who sat for the American Society for Clinical Pathology Board of Certification examination: graduates' perspective

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Key words: COVID-19, survey, ASCP BOC, MLS programs, education, pandemic

Abbreviations: ASCP BOC, American Society for Clinical Pathology Board of Certification; NAACLS, National Accrediting Agency for Clinical Laboratory Science; ABHES, Accrediting Bureau of Health Education Schools; CAAHEP, Commission on Accreditation of Allied Health Education; IT, information technology

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ABSTRACT

Objective: Students in health profession education programs were severely affected by the COVID-19 pandemic at both didactic and clinical training levels. The purpose for this American Society for Clinical Pathology Board of Certification (ASCP BOC) study was to determine the impact of the COVID-19 pandemic on graduates. This study represents the perspectives of laboratory professional graduates who sat for the BOC certification in their respective professional disciplines.

Methods: A survey was sent to all graduates from the National Accrediting Agency for Clinical Laboratory Science (NAACLS), Accrediting Bureau of Health Education Schools (ABHES), and Commission on Accreditation of Allied Health Education Programs (CAAHEP) accredited programs who sat for the ASCP BOC examination in 2020 and 2021 to determine the impact of COVID-19 on laboratory professional graduates during the pandemic.

Results: A total of 180 graduates responded to the survey. The majority of graduates indicated that at least 1 didactic program component was shifted to an online system during the pandemic and that both clinical and nonclinical student laboratories were affected. Although program completion for most graduates was not delayed, one-third of graduates delayed taking their respective BOC exam. Due to the lack of knowledge application through practical hands-on laboratory experience in their educational programs, graduates reported feeling a lack of readiness with regards to preparing for the national certification examination as well as for employment.

Conclusion: The study results showed the pandemic greatly impacted the education experience and readiness for the ASCP BOC examinations for graduates. Factors such as the absence of in-person learning and hands-on experience—both crucial aspects in laboratory training—and the ripple effects as a result of the pandemic, such as job loss, financial constraints, and health concerns, contributed to the decreased quality of education for graduates.

Introduction

In general, college students' educational experiences were significantly affected by the COVID-19 pandemic due to mandatory precautions to prevent transmission of the virus. In addition, the education of health care professional students was also challenged by delays or abbreviated clinical components of the curricula due to the stress on medical professionals and facilities to assist with the care of COVID-19 patients. Thus, students enrolled in National Accrediting Agency for Clinical Laboratory Science (NAACLS), Accrediting Bureau of Health Education Schools (ABHES), and Commission on Accreditation of Allied Health Education Programs (CAAHEP) accredited educational programs were no exception, experiencing severe consequences on their personal lives and educational outcomes. In a previous publication,[1] the impact of the COVID-19 pandemic on graduates who sat for the American Society for Clinical Pathology Board of Certification (ASCP BOC)

certification in their respective professional disciplines was discussed from the perspective of program directors. The purpose of this article is to reveal the results of the survey that was distributed to graduates of the accredited programs to gain insight into their unique perspectives.

Methods

Two surveys were conducted by the ASCP BOC Research and Development Committee to determine the impact of COVID-19 on health care graduates and education programs during the pandemic. One survey was sent to all program directors from accredited programs whose graduates are certified by the BOC, and a second survey was sent to graduates who sat for the ASCP BOC examination in 2020 and 2021. The surveys were distributed as electronic invitations via Key Survey (an online survey tool) on June 6, 2022. A reminder was sent on July 6, 2022, and the surveys were closed on October 11, 2022. A total of 180 graduates and 201 program directors responded. This article reports on the graduates' responses. A separate article reported on the program director responses.[1]

Results

Demographics

Of the 180 responses received, the majority (84.4%) were graduates of university-based programs (**TABLE 1**). By program, the majority were medical laboratory scientist (MLS) graduates (52.8%). Respondents represented programs in 46 states plus the District of Columbia and Puerto Rico, with 1 international respondent.

Hospital programs affiliated with the graduates who responded to the survey included MLS, technologist in molecular biology, histotechnician, cytologist, and specialist in blood banking. Graduates associated with universities represented the following programs in descending order of percentages of respondents: MLS, medical laboratory technician (MLT), pathologists' assistant, phlebotomy technician, histotechnician, cytologist, and technologist in molecular biology.

Given the low numbers of non-MLS and hospital graduates, the majority of the data for this article is reported for the entire sample without separating by program or affiliation. Overall data also showed that graduates across programs and affiliations faced similar educational experiences during the COVID-19 pandemic, which provided more confidence to report on the group as a whole.

Impact of COVID-19 on Nonclinical Student Laboratories

The impact on nonclinical student laboratories (laboratory sessions occurring outside the clinical setting) was consistent across all programs (**FIGURE 1**). The most common impacts were moving student laboratory exercises to an online format (55.6%), replacement with simulation or other activities (28.9%), and cancellation (26.1%). Specific comments referenced shortening the time in laboratories; splitting classes into smaller groups; requiring masks, cleaning, and social distancing; and using a hybrid of online, paper-based, and in-person formats.

TABLE 1. Demographics of respondents (n = 180)

Program affiliation	Percentage of respondents
Hospital programs	15.6
University programs	84.4
Program	
Medical laboratory scientist	52.8
Medical laboratory technician	35.0
Other[a]	12.2

[a]*Other programs included cytologist, histotechnician, pathologists' assistant, phlebotomy, specialist in blood banking, and technologist in molecular biology.*

Graduate Voices—Student Labs

"No hands on time"/"No anatomy lab, class not as comfortable with anatomy in autopsy."/"Could not screen slides"/ "Inability to screen slides as that is our area of study"

"It was difficult to learn the material by myself since many professors provided just powerpoints, and labs were cancelled so it was difficult to relate concepts to the application of them in the real lab."

"Everything was moved to online class including labs. I don't feel like I was adequately prepared for hands on experience in the lab."

"Having to learn online made it more difficult to learn and retain information."

"My instructors did the best they could with the situation at hand and all its unknowns. I learn best 'hands on', as did most of my cohort. I'm not sure what else could have been done at the time to help us. That being said, going into clinicals and the workforce was nerve wracking after spending our second year in front of a computer. I wish my boss would acknowledge that I came to my job as a COVID graduate. It would have helped."

"The pandemic overall affected my learning environment in a very negative way. My lectures were less engaging, professors were less accessible. A huge problem was in-person labs were moved online, yet those manual skills that were supposed to be taught and practices were still expected of the students. Without that valuable time for practicing the manual work, those skills did not have a chance to develop until the clinical rotation. Along with that, many of the lecture topics did not fully make sense until testing and using the knowledge in person on actual specimens."

FIGURE 1. Impact on nonclinical student laboratories.

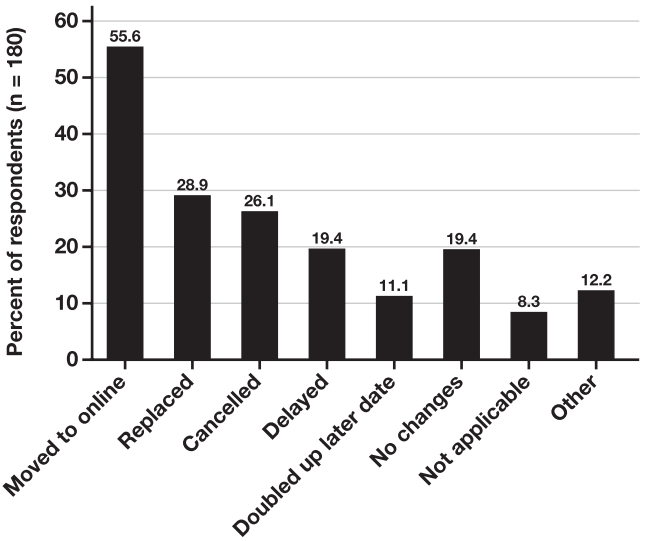
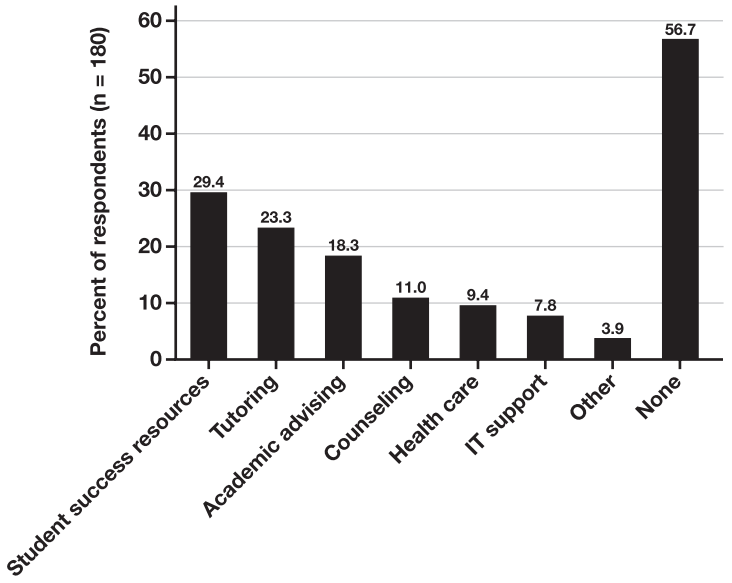


FIGURE 2. Impact on academic support.



Academic Support

The majority of respondents (56.7%) reported no difficulty in receiving academic advising, counseling, student success resources, tutoring, information technology (IT) support, or health care services (**FIGURE 2**). However, nearly a quarter to a third of the students reported difficulty accessing student success resources, such as time or stress management and examination taking skills (29.4%) and tutoring (23.3%), compared with the other categories.

Graduate Voices—Support

"Massive layoffs affected teaching staff, online learning program & material, and overall support of the program."

"All extra curricular educational activities outside of clinical rotations were cancelled - such as class parties, site visits to the state lab, and in person conferences."

"All libraries were closed. That's where I would go to study for an upcoming test."

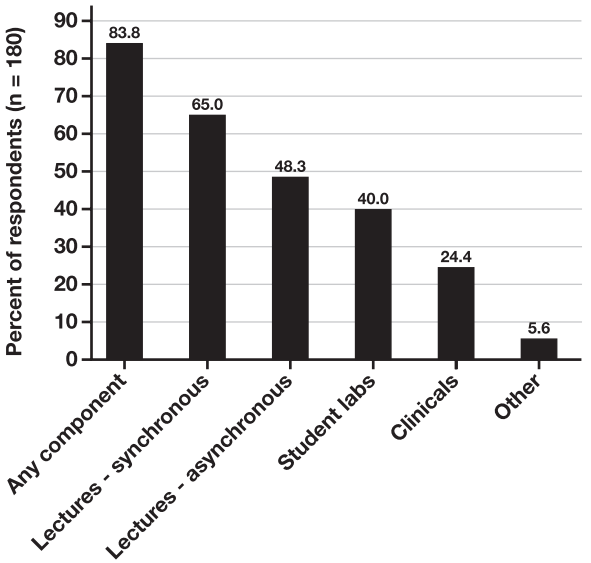
"IT issues were difficult to resolve or took longer to resolve because of lack of staff at university, program was asked to disband because of funding issues."

"Delayed contact with instructors when we didn't understand part of the curriculum"/ "Some teachers preferred to answer questions only online through email. Email conversations sometimes are not answered timely and information can be unclear."/ "Not being able to get the help I needed through online. Slow response times. Online lectures were not followed through."

"Slow response times from advisors."

"Very little resources. Accomodations didn't last but one day."

FIGURE 3. Shifting program component to online.



"Lack of educational supplies ie, books, paper, ink, communication with instructor's and class mates while struggling with clinical subjects."

"Lack of resources offered by university, lack of communication from university."

Shifting to Online

The majority of respondents (83.8%) reported a shift of at least 1 program component to an online system during the pandemic (**FIGURE 3**). The curriculum component most often affected was face-to-face lectures, which were changed to either a synchronous online format (65.0%) or asynchronous delivery arrangement (48.3%). Student laboratories were also affected, with 40.0% of respondents reporting a shift to an online

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design. Additional comments revealed that the students also experienced shortening of courses and hybrid structures.

Impact on Classes

The most commonly reported specific impact on classes was more time spent online (70.6%) followed by the need to adjust personal schedules to accommodate online/hybrid learning (36.1%) and smaller class sizes (21.7%) (**FIGURE 4**).

Graduate Voices—Class Impact

Common theme: "Laboratories cancelled, lectures were PowerPoint only/ face-to-face lectures to pdf files we had to learn ourselves"/ "'Lectures were read the PowerPoint and read the chapter"/ "Lectures stopped after 1 try going online."/ "When covid happened the school was closed down except for online, we did our reviews and finals one semester all online"

"Recorded lectures same length as F2F ones but teacher talked entire time, no pauses. Note taking was much more tedious as we waded through three hours of non-stop talking. No whiteboard drawings to assist lecturer in explaining concepts. Lecturer unable to look at class for confirmation of comprehension. Large delay in obtaining material, quizzes, exams."

"I feel I didn't get the hands on at the end to put all of my education together. Learning from the pathologists on live cases would have greatly improved my confidence entering the workforce. We were unable to practice screening on our 'unknown' boxes for practice or ask questions to our instructor as we would have gone through that process. We were unable to learn from each other as we were separated. We were not able to utilize the mentoring program to work on live cases. We had to go home for 6 weeks."

"My program suffered from layoffs one month after my class started. We had all new advisors and directors. They did not know how to operate Blackboard and our education material, homework, and quizzes were often released late or incomplete. We constantly were having technical difficulties and behind in our studies, struggling to stay caught up as the timelines for tests would not change even if we got the material a week or two late. The program was often very frustrating and unnecessarily stressful due to these issues. The program eventually gave us a partial refund, acknowledging some of the failures that occurred."

"I felt like I was not able to grasp the information to pass on the first attempt due to online format of learning."

"No one was prepared for Covid - the college or the instructors and the instructors even apologized because they said we really didn't get enough time in the classroom because online was difficult for the instructors how can you be ready or even prepare yourself for something like that."

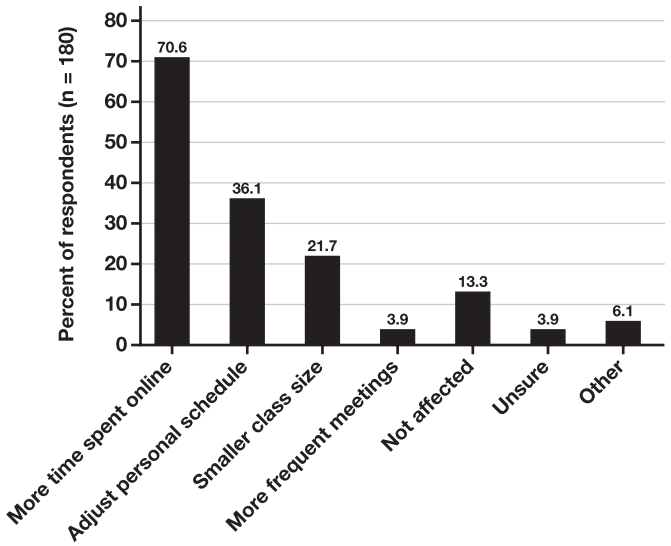
"Online classes ruined my experience at the program. Everything was delayed and when online classes started the program was saying we had to move on quickly because they had another group coming in. My clinical rotations were delayed and shorten by half each one. Online classes can't replace face to face learning."

"Personally I feel as if the program itself was not affected in any major way but wearing masks has made it hard to understand professors and trainers at times. Also splitting up my [sic] made it hard to understand my professor because he was being recorded live from a TV since he was not present in my room. Additionally, there was a reduction in the amount of places we could go within the school or hospitals we trained in to study due to capacity rules. All in all, it was a minor effect."

"Switched schedule around to make it more condensed."

"The hybridization of the learning format was an unexpected HUGE positive impact of the pandemic. I found I was able to pay much more attention and retain more information by attending lectures virtually. The hands on lab work was of course essential, and the need to consolidate and intensify those dates were a challenge. But the overall hybridization format was actually really great."

FIGURE 4. Impact on classes.



Grades and Grading

Only 6.7% of respondents reported changes to the grading scale (**FIGURE 5**). Half of respondents (50.0%) believed the pandemic had no impact on their own grades; however, 21.7% reported a negative impact.

Program Completion Timeline

Overall, 78.9% of respondents completed their programs on time and were not delayed due to the COVID-19 pandemic (**FIGURE 6**). The majority of delays, however, were related to clinical placements, as reported by 15.6% of respondents.

Graduate Voices—Program Completion Time

"All classes delayed due to fighting for class spots in smaller classes."

"Completed as scheduled, but intro summer classes were moved to following summer and forced students to take higher level classes first."

"Completed but I felt I was rushing a lot."

"Completion two weeks delayed."

"Program completion date delayed due to hospitals not wanting to accept students. Delayed to the point I was dropped and worked with Program Director to reinstate me to finish my Clinical Rotation."

"At the time, information was relayed to us (students) very poorly as to whether or not we'd graduate."

"Schooling got pushed back a whole year than what I was originally enrolled for, I originally had a part-time job so I could spend more time studying for school but once I got kicked out of all my classes because of COVID I had to get a full-time job and because of that I was full-time working and trying to be a student which made it very difficult to have any time for myself or to study."

FIGURE 5. Impact on grading scale and grades.

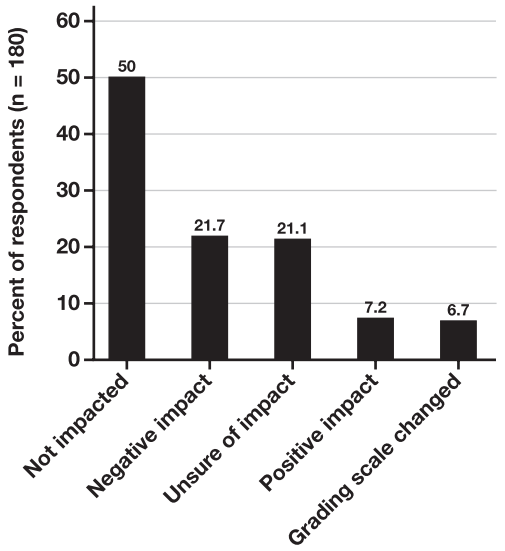
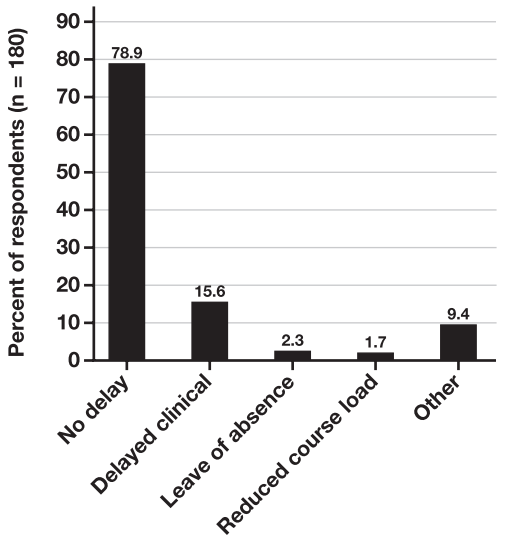


FIGURE 6. Impact on program completion.



Impact on Clinical Rotations

Nearly half of the respondents reported that their clinical rotations were unaffected by the pandemic. When clinical rotations were affected, the most frequent response was that they were shortened (22.8%); however, approximately 17% of the respondents also reported that their clinicals were either delayed, cancelled, or replaced with simulation (**FIGURE 7**). The specific rotations affected were also fairly evenly distributed. Notably, approximately 30% of the respondents reported that microbiology and blood banking clinical rotations were affected by the pandemic (**FIGURE 8**). Forty-eight percent of respondents indicated that none of their clinical rotations were affected.

Graduate Voices—Clinical Rotations

Common theme: clinical rotations done online, no hands on experience in a lab"/ "My bloodbank clinical was cancelled and moved to campus during spring of 2020"/ "Canceled due to labs being short staffed and not able to take students"/ "moved online, all classwork"

"Some hospitals rejected students for clinical rotations, and other students had to go to other states to do clinical rotations. At the students expense."

"Very chaotic, did not know location/dates/times until a month or sometimes weeks before rotation."

"Specialties mostly HLA and flow cytometry were impacted"

"Unable to go on procedures or signouts with pathologists. No longer able to meet with all students (4 of us), unable to continue to learn lab prep area. Unable to screen slides"

"Anything with patient interaction was highly limited. Little interaction on how to collect certain specimens from patients in person."

"They let us graduate in time but we were left to fend for ourselves as we navigated getting our first jobs."

"The program director of the school I attended was absolutely unrealized about her expectations during Covid. Her changes to clinical were unreasonable and she did things that she said were agreed to but absolutely were not agreed to by the students in the program."

FIGURE 7. Impact on clinical rotations.

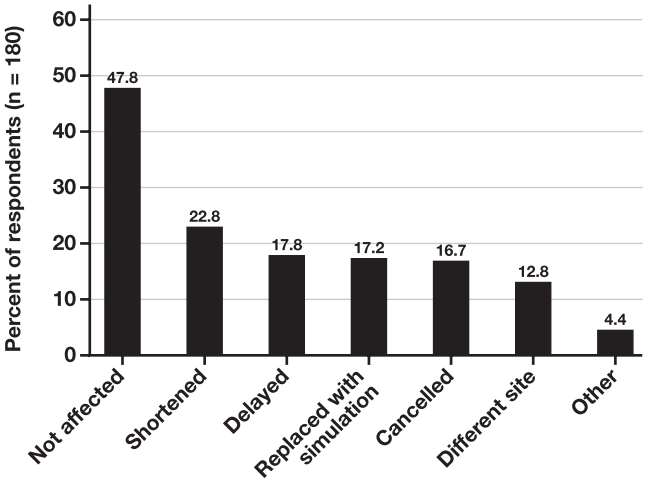


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FIGURE 8. Specific rotations affected.

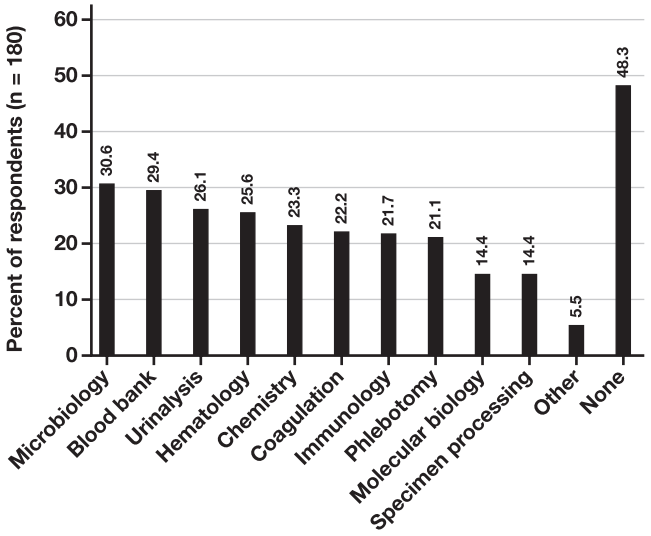
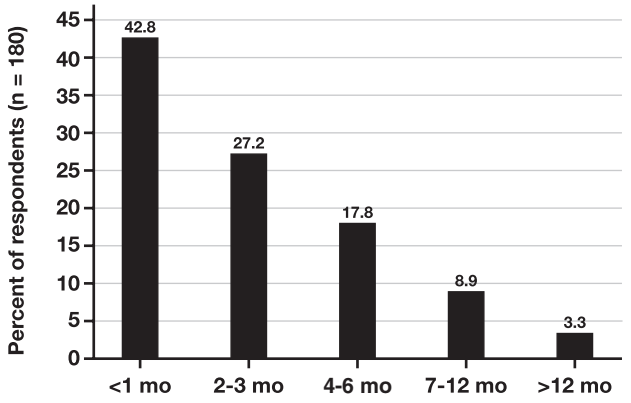


FIGURE 9. Time between program completion and examination.



Impact on Certification

Overall, 32.2 % of respondents reported a delay in taking their certification examination due to COVID-19 (MLS, 29.5%; MLT, 41.3%; other,18.2%) (**FIGURE 9**). The most commonly cited reason for a delay was feeling unprepared for the exam (17.2% of respondents), with less than 7% citing financial issues, difficulty scheduling the exam site, delayed program completion, or other reason. Most respondents (67.8%) reported no impact on the selection of their preferred date for the exam or their desired testing location (70.0%). The majority of respondents (42.8%) took their exam within 1 month of program completion.

The majority of respondents (76.7%) reported passing their certification exam on the first attempt (MLS, 80%; MLT, 73%, other, 72.7%). Respondents were almost equally divided between believing that COVID-19 did not affect their exam scores (48.9%) and did have a negative impact (41.1%) (see **TABLE 2**).

TABLE 2. Percentage of respondents' perception of COVID impact on exam scores

Impact	MLS (n = 95)	MLT (n = 63)	Other (n = 22)	Total (n = 180)
Not affected	48.4	52.4	40.9	48.9
Negative impact	42.1	33.3	59.1	41.1
Positive impact	9.5	14.3	0	10.0

Graduate Voices—Certification

"Honestly, this is a program that you've got to take in person. By moving online half way through the semester, we missed out on vital information that by doing so, set us up for failure due to no fault of our professors or program director. I feel that if we had stayed in person, I would have passed my exams. I believe that that this is evidently shown by the graduating class of 2022 having a higher certification exam pass rate."

"I believe I could have passed the ASCP exam if Covid didn't come around right in the middle of my school year. Covid canceled all classes in March 2020-July2022. I was at the top of my class and on my attempt of ASCP I made a 394."

"Was unable to take exam during times given as I got COVID and became very Ill I could not study I could not drive so I just gave up. Still a big regret. Because I worked hard and I was a good phlebotomist"

"Due to being dismissed from clinical rotations, I believe this impacted my preparedness for the BOC exam."

"I was not able to take the exam for 7 months after our clinical rotations were canceled. I was never alowed to finish my rotations and never received the practical training in microbiology, blood bank, or hematology. This delay made it near impossible for me to pass the ASCP exam. I'm still unregistered and unable to get a job in a general lab because I'm unregistered."

"Due to being dismissed from clinical rotations, I believe this impacted my preparedness for the BOC exam."

"As for the exam I had no problems scheduling or taking my exam. I know many MLT & MT students at my hospital had to wait months to take their exams though."

"Despite not having as much hands-on experience in my micro rotation my last semester, I got a good enough understanding to pass the ASCP generalist exam."

Personal Impact

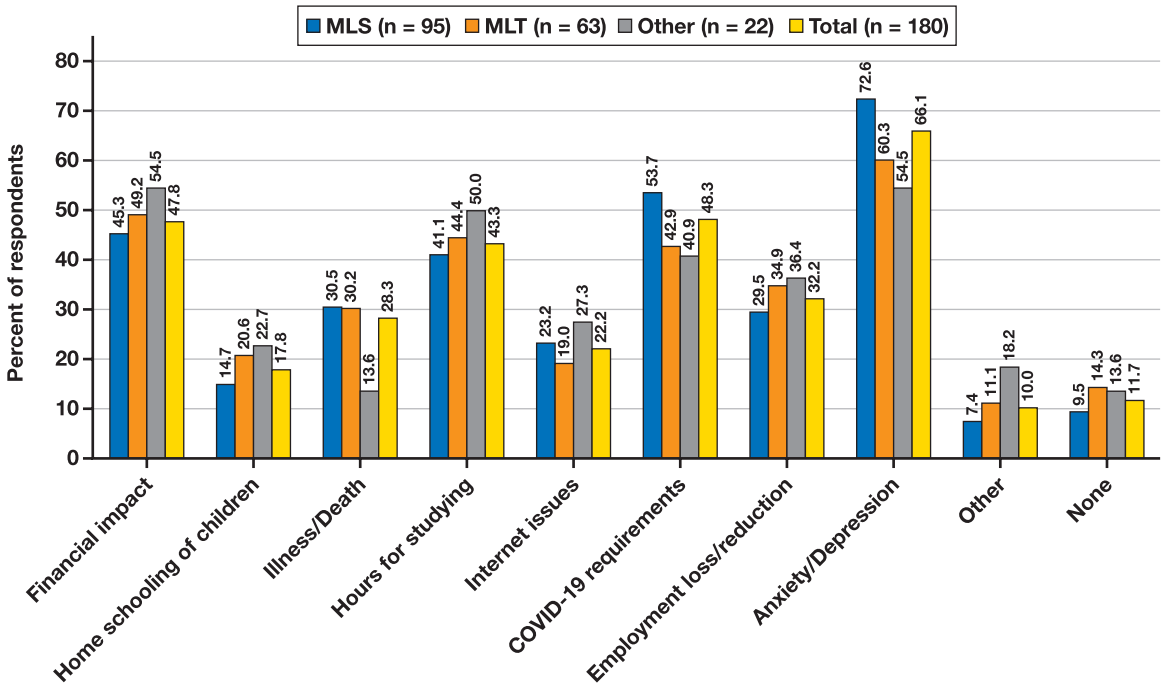
Respondents reported several personal consequences of the pandemic that affected their ability to complete their program, take their certification exam, and/or pass their examination (**FIGURE 10**). Respondents were nearly equally divided in financial impact of the pandemic, with 52.2% reporting no impact. Financial consequences included the ability to take the certification exam (15.6%), pay for school (23.3%), and complete the program as intended (10.6%).

The most frequently cited impact was general anxiety/depression (66.1%) followed by COVID-19 requirements (masking, social distancing, vaccination, etc) (48.3%) (**FIGURE 10**).

Graduate Voices—Personal Impact

Common theme—financial difficulties: "The program was already fast paced but we had to do even more with less time and we had also lost our

FIGURE 10. Percent of respondents reporting negative personal impacts.



prn positions due to furlough which affected us tremendously."/"Ability to take care of personal bills and expenses"/"Had to drive further to get to school, this affected my ability to make money."/"Homeless while attempting MLT Program, trying not to fail program."/"No tuition assistance offered from my work due to COVID."/ "Spouse's concern/anxiety that I wouldn't graduate on time and get a job."

"It was just hard. Would have given up but had a great director who understood that everybody was struggling and just accommodated each one accordingly."

"More hours worked due to Hospital Staffing Shortages. Higher levels of stress due to Hospital work & Burnout"/ "Extra workload due to pandemic and staff shortages"/"I was a FT employee in a Hospital and had to balance school, children, and home."/" Increased demands at place of employment."

"Working using my MLT trying to get my MLS. But my hours went sky high into overtime making it very hard to study or prepare for MLS exam. I felt like I should do all I could for society at my job and was just absolutely emotionally and physically drained every day after work. I was working in a 1 man lab at a stand up ER. So I had lots of patient contact, and little rest."

"Uncertainty. We always had to worry about whether or not we were going to be suddenly switched to online format if one of us got COVID."

"Increased workload in lab testing meant students were more learned in to help completing workload in a timely manner. Having safe spaces to meet with cohort members to study was impacted, our program coordinator was only able to help us only after studying together outdoors was no longer feasible due to the cold or heat. Generally stressful environment."

"So busy I couldn't truly be immersed in just what I was learning. I had to multitask the work for school and at the hospital in order to continue doing the program."

"There were times I wondered if I wanted to go through with being in the medical profession. It was just hard. Got a job right away but the world was too much of a burden for a new hire."

"Because of COVID-19, laboratory facilities question my and other students lack of understanding. I have failed board two times and preparing for my third attempt. I have been denied raises and promotion due to facilities not understanding the effects COVID-19 has caused on students' success. There were no accommodations from school on our success to complete and graduate during a Global Pandemic and neither from Pearson when taking a BOC exam."

"I also experienced the loss of a parent so it has been very difficult with my anxiety and test taking."

"mental/physical/emotional/educational depletion due to circumstances"

"I got through all of it eventually but was really peeved when my hospital I was working for and many of the ones I have heard of paid all sorts of OT benefits to nursing and respiratory staff that was working extra. When although I had a lot of patient exposure, and was running testing the lab ended up overlooked. I wish that more governing bodies would help shine a light on the lab rather than laboratorians being the introverted meek people in the workforce that take whatever including poor pay."

"The program I graduated from was great in handling Covid while still making sure we were learning what we needed to. I am not a very good test taker and suffer from test anxiety which is why I made the mistake of procrastinating to take the certification exam. I also had anxiety during test because we had to wear a mask covering, big headphones to block out sound and were put into cubicles so I felt I couldn't concentrate and focus on test. I do not want to give up on getting my certification so I have been getting back into studying and saving up to afford the exam and then retaking it."

"There wasn't enough time to review for the exam. Originally there was talk of getting together, being able to come in for extra labs, and reviewing for the certification exam. Our lab director had the professor quit suddenly due to issues with homeschooling and online bullying which made her have more classes to take on and no time to review. I personally had homeschooling with my son and all the struggles that went with that. I got Covid during my internship and was lucky enough to have an understanding lab manager. The Covid brain fog is real. I just really feel if we could have even gotten together and reviewed for the certification, that would have really made things less stressful."

Discussion

The purpose of this article was to provide a descriptive analysis of laboratory professional graduates' perceptions of the impact of COVID-19 pandemic on their performance in medical laboratory science programs, the ASCP BOC examinations, and their personal lives. Although the low number of respondents (n = 180) was disappointing, the data generated from the survey verified the data compiled from the published study of the program directors' perspectives and in some cases provided a more accurate viewpoint of the situation.

The demographics of the 2 groups of respondents of the surveys, the directors and graduates of the laboratory science programs, were similar. The majority (over 80%) of the respondents of both groups were related to MLS and MLT programs; other programs comprised 12.2% of the respondents who were graduates of these programs and 13% who were program directors. Both groups of respondents reflected a wide representation from more than 40 states.

Within MLS educational programs, the COVID-19 pandemic's greatest impact may have been on the growth of psychomotor skills. Basic, intermediate, and advanced laboratory psychomotor skills are developed in a stepwise fashion through nonclinical student laboratory sessions, most often observed in university-based programs, and more importantly, through clinical rotations. Regarding changes in the delivery formats of student laboratory sessions due to the pandemic, 55.6% of graduates and 56.1% of program directors reported a change from face-to-face to an online format. Substitution of the hands-on practice of laboratory medicine and pathology laboratory skills with online sessions was indeed challenging and varied based on the technology and resources available to the program instructors. Based on the students' perspective, only 29% reported that the student laboratory sessions were replaced with simulation, whereas 60% of university program directors reported this format change. This discrepancy may be the result of the variable interpretation of the definition of simulation. However, cancellation of the nonclinical laboratory sessions was reported by a higher number of graduate respondents (26%) compared with 15% of program directors. Thus, students missed out on opportunities to develop and practice psychomotor skills prior to clinical rotations. Through survey commentary, graduates addressed the ineffectiveness of the online format for the instruction of laboratory skills on comprehension and retention of lecture information and applying concepts learned in the classroom to the "real laboratory." Further, this change from student laboratory sessions to an online format resulted in students feeling less prepared for the clinical hands-on experiences.

A similar pattern of responses between the 2 groups was also observed for the impact on clinical rotations. Almost half of the program graduates reported that their clinical rotations were unaffected, although 17%, 23%, and 17% indicated that the rotations were cancelled, shortened, or replaced with simulations, respectively. In contrast, less than 10% of the university-based program directors reported that their clinical rotations were unaffected. Both groups of respondents, however, indicated that the blood banking and microbiology clinical rotations were more negatively affected than other areas. Comments from the graduates emphasized the negative effect on the learning environment from losing the valuable time to practice manual skills and procedures. However, the graduates expressed frustration that the supervisors in the student clinicals still held the expectation that these students possessed these skills. Interactions with the pathologists and patients were severely affected.

Notably, several graduates commented on the negative effect from the lack of exposure to laboratory practice on employment performance postgraduation. Comments suggested that their new jobs were "nerve wracking" and that they were "left to fend for ourselves." One respondent reported that no opportunity was provided to complete clinicals in microbiology, blood banking, and coagulation, whereas 1 graduate stated that they "never stepped into a laboratory" for in-person student laboratory sessions or clinical rotations. One respondent expressed the desire for those in the clinicals to have more patience and a better understanding of the limitations in the educational experiences of those graduates during the COVID-19 pandemic.

Several of those who graduated from laboratory medicine programs during the pandemic and did not pass the ASCP BOC certification examination blamed the change to the online format of the educational components and lack of clinical rotations for the failures. Indeed, the ASCP BOC annual statistics indicated a noticeable decrease in the average pass rates for NAACLS first-time examinees in both 2021 and 2022 compared with 2019 and 2020 statistics. In 2019[2] and 2020,[3] the pass rates for NAACLS first-time examinees for the MLS were 85% and 83%, respectively; MLT examinations were 83% and 84%, respectively. For the same examinations in 2021[4] and 2022,[5] the pass rates were 79% and 78% for MLS examinations and 78% and 78% for MLT examinations. Although a decrease was observed over 2019-2022, it is worth noting that the average difference in effect was minimal between the years. More information on test scores and pass rates for MLS, MLT, and other programs can be found on the ASCP BOC website.[2-5]

Delay in taking the certification examination was reported by 32% of the graduates who responded to the survey, citing unpreparedness as the most common reason; over half of the program directors reported that their students delayed taking the certification examination. The respondents who were graduates did not cite delays in the BOC response to questions as a major issue; however, the program directors provided this as a cause in the delay of their students to register for the examination. A previous study showed that longer delay between finishing the program and taking the exam resulted in a significant decrease in examination scores.[6]

Comments from graduates on the personal impact of the pandemic provides a more accurate account than anecdotal reports. The greatest negative impact from the pandemic, as reported by 66% of the graduates, was experiencing general anxiety and depression while

completing the educational program. Other negative effects that most likely contributed to the anxiety and depression included adhering to COVID-19 requirements, financial problems, changes to employment, and home schooling of children. Although some working students experienced the loss of PRN positions to furlough during the pandemic, others were overworked in laboratory positions leaving little time for studying (approximately 40% of respondents).

Conclusions

The results from graduates completing the survey on the impact of the COVID-19 pandemic were similar to those of program directors and thus helped to verify the survey results. Emphasized in the data and comments from the students was the feeling of lack of preparedness with regards to registering for the national BOC examination as well as for employment. Graduates who lacked practical hands-on laboratory experience in their educational programs expressed the desire for empathy as they maneuver through their first professional experiences with limited psychomotor skills. Longitudinal studies on the long-term effects of the pandemic on employment performance will be useful in planning for curriculum changes imposed by disasters of this nature in the future.

Conflict of Interest

The authors have nothing to disclose.

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OVERVIEW

A meta-analysis of urinary transferrin for early diagnosis of diabetic nephropathy

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Key words: diabetic kidney disease; urine; transferrin; diagnostic value; meta-analysis; early diagnosis

Abbreviations: CKD, chronic kidney disease; DN, diabetic nephropathy;ESRD, end-stage renal disease; GFR, glomerular filtration rate; eGFR, estimated glomerular filtration rate; Tf, transferrin; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; UAER, urinary microalbumin excretion rate; UACR, urinary microalbumin/creatinine ratio; TP, true positive; FP, false positive; FN, false negative; TN, true negative; PLR, positive diagnostic likelihood ratio; NLR, negative diagnostic likelihood ratio; DOR, diagnostic odds ratio; T2D, type 2 diabetes; ROC, receiver operating characteristic; LR, likelihood ratio; prob, probability; pos, positivity; neg, negativity;DM, diabetes mellitus;NO, normal albuminuria;MI, microalbuminuria; MA, macroalbuminuria; NA nonapplicable

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ABSTRACT

Objective: To assess the diagnostic value of urinary transferrin (Tf) in early diabetic nephropathy (DN) to propose a more sensitive and noninvasive biomarker for screening and monitoring DN in clinical practice.

Methods: We searched 3 databases from their inception to May 2023, to identify studies investigating the diagnostic value of Tf in patients with DN. Meta-DiSc software, version 1.4, and Stata software, version 15.1 (StataCorp) were used to conduct a meta-analysis and evaluate the diagnostic accuracy of urine Tf levels for DN.

Results: The meta-analysis included 6 relevant studies investigating the diagnostic value of Tf level for DN. Urinary Tf as a diagnostic marker demonstrated a combined sensitivity of 0.82 (95% CI, 0.71-0.89) and specificity of 0.88 (0.84-0.92). the positive diagnostic likelihood ratio was 7.07 (4.57-10.93), the negative diagnostic likelihood ratio was 0.20 (0.12-0.35), and the diagnostic odds ratio was 34.49 (13.61-87.44). Also, the area under the receiver operating characteristic curve was 0.92 (0.89-0.94), indicating that urinary Tf has a decent discriminative ability in diagnosing DN.

Conclusion: Tf level is a valuable biological marker for early diagnosis and monitoring of DN in clinical practice. It has statistically significant predictive value for patients in the early phases of DN.

Introduction

Patients with diabetes are approximately twice as likely to develop chronic kidney disease (CKD) as those without diabetes.[1] Diabetic nephropathy (DN) is characterized predominantly by persistent proteinuria, progressive decline in renal function, and elevated serum creatinine levels. Inadequate or ineffectual treatment may lead to the development of chronic renal failure, acute renal failure, end-stage renal disease (ESRD), and various nephrotic syndromes, which may result in death due to the protracted course of the disease.[2]

Microalbuminuria serves as an early diagnostic indicator of DN.[3] However, extensive research findings have demonstrated the rising incidence of nonproteinuric CKD (estimated glomerular filtration rate [eGFR] ≤60 mL/min/1.73 m^2 without proteinuria) among patients with diabetes.[2-4] Therefore, early detection of these patients with nonproteinuric CKD among the patient population with diabetes is crucial.

Blood urea nitrogen (BUN), creatinine, eGFR, and proteinuria are currently the leading indicators for clinical diagnosis of DN and severity assessment.[5,6] However, to a certain extent, these markers are not optimal for assessing early DN. The findings of another study[7] revealed that 20%-40% of patients with diabetes experienced a decline in GFR before the detection of albuminuria. In this 10-year follow-up study, Fioretto et al[7] found that 30%-45% of patients with microalbuminuria progressed to having macroalbuminuria. In total, 30% of patients returned to normal levels of urine protein, and 30%-40% of patients continued to have microalbuminuria.[7]

In clinical practice, the albumin-to-creatinine ratio is the most common urinary biomarker for diagnosing patients who have diabetes with co-occurring renal disease. Also, eGFR is a crucial indicator for evaluating kidney function and determining the stages of kidney disease. Both markers are important tools for patient management because they provide an estimation of kidney function, which helps in determining the CKD stage and in guiding therapy.[8] However, the sensitivity of these markers in detecting subtle changes in renal function is limited.[9] Although renal biopsy remains the criterion standard for

diagnosing DN, its invasiveness limits its use in routine clinical practice. Therefore, it is necessary to investigate more sensitive and noninvasive markers in this discipline.

DN predominantly affects the glomeruli, renal tubules, and renal interstitium with pathological changes. Glomerular injury causes an increase in the urinary excretion of plasma proteins,[10] such as urinary transferrin (Tf), urinary ceruloplasmin, and urinary immunoglobulin G, which serve as markers of glomerular injury. The proportional increase in urinary Tf, urinary immunoglobulin, and urinary ceruloplasmin levels suggests their potential as early biomarkers for DN.[11] Early-stage DN is characterized by increased glomerular permeability, which results in albumin and other plasma proteins (such as Tf) leaking into the urine from the plasma.

Despite having a comparable molecular weight to albumin, Tf has a higher isoelectric point.[12] Due to alterations in its charge, the charge barrier of the glomerular basement membrane weakens in the early phases of DN. Consequently, Tf encounters diminished repulsive forces, facilitating its leakage and providing a more accurate reflection of charge barrier damage.[12] Injuries to the renal tubules are another vital characteristic of DN.[13] Before the advent of microalbuminuria, markers of tubular damage might have been elevated in patients with diabetes.[14] Kanauchi et al[15] reported a positive correlation between tubular dysfunction and urinary levels of microalbumin and Tf in patients with DN.

Hence, the objective of this study is to conduct a meta-analysis to assess the screening efficacy of urinary Tf in early DN. Our aim is to propose a highly sensitive and noninvasive biomarker for the screening and monitoring of DN in clinical practice.

Methods

This meta-analysis was performed according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[16]

Retrieval Strategy

Using predefined keyword search terms, we conducted a comprehensive literature search in PubMed, Cochrane Library, and Web of Science. The search terms used included "diabetic nephropathy," "diabetic kidney disease," "DKD," "DN," "transferrin," and "TRF." To accommodate the specific requirements of each database, the search strategy was refined using a combination of subject terms and free terms. The literature search was conducted from the inception of the databases until May 2023, with no language restrictions.

Literature Inclusion and Exclusion

The study inclusion criteria were defined as follows: adult participants (aged ≥18 years) with diabetes mellitus, DN staging based on urinary microalbumin excretion rate (UAER) or urinary microalbumin/creatinine ratio (UACR), and investigation of the predictive value of urinary Tf in DN using receiver operating characteristic (ROC) curve analysis. There were no restrictions on language, publication year, or publication type. The exclusion criteria were as follows: comments, letters, or conference abstracts; studies lacking true positive (TP), false positive (FP), false negative (FN), and true negative (TN) results; participants with severe infection, diabetic ketoacidosis, primary kidney disease, malignant tumors, or drug-induced renal injury; and duplicate publications.

Data Extraction and Literature Quality Assessment

According to the inclusion and exclusion criteria, 2 independent researchers extracted the following data from each included study: first author; publication year; study location; sample size; standards and classification of DN; method of urinary Tf detection; Tf threshold; number of TP, FP, TN, and FN occurrences; and cutoff value of Tf. Group discussions involving a third researcher resolved any disagreements between the 2 researchers. The quality of the included studies was evaluated using the QUADAS-2 scale,[17] which consists of 14 evaluation items, each with 3 response options (yes, no, or unclear). The 2 evaluators evaluated each study independently, line by line, and discussed any disagreements with the third researcher.

Statistical Analysis

Meta-Disc software, version 1.4, and Stata software, version 15.1, were utilized in this investigation for diagnostic meta-analysis.[18,19] Several indicators, including sensitivity, specificity, positive diagnostic likelihood ratio (PLR), negative diagnostic likelihood ratio (NLR), diagnostic odds ratio (DOR), and area under the curve (AUC), were used to evaluate the diagnostic value of urinary Tf for DN.

First, to assess heterogeneity among the included studies, we used Meta-Disc, version 1.4, employing an ROC scatterplot and Spearman correlation coefficient method. Next, using Stata version 15.1, the effect size, sensitivity, specificity, PLR, NLR, DOR, AUC, and their respective 95% CI values were subsequently analyzed. If there was heterogeneity between the studies ($P < .05$ or $I^2 > 50\%$), a random-effects model was used; otherwise, a fixed-effects model was used. Finally, publication bias was assessed via Deeks funnel plotting; $P < .05$ was considered statistically significant.

Results

Literature Screening, Basic Characteristics, and Quality Evaluation

We initially retrieved 115 articles from the databases mentioned herein. Ultimately, 6 studies were included in the meta-analysis after screening (**FIGURE 1**).[20-25] These 6 cross-sectional studies, involving 1274 participants, were published between 2017 and 2022. Two of the papers were limited to the microalbuminuria group, and the remaining 4 included the microalbuminuria and macroalbuminuria groups. The enzyme-linked immunosorbent assay or the immunoturbidimetric method was used to detect Tf in urine. DN was diagnosed based on UAER or UACR criteria. For data extraction, pertinent information was recorded, including the first author; publication year; country; total sample size; number of cases in the DN group; number of control individuals; number of TP, FP, FN, and TN occurrences; DN diagnosis; Tf threshold; and urine Tf detection method (**TABLE 1**).[20-25]

We evaluated the 6 articles included in this study using the QUADAS-2 scale diagnostic quality evaluation form. There are a total of 14 items on the scale. Each item was assessed based on its applicability to the included studies, using the 3 response options "yes," "no," and "unclear." **TABLE 2** displays the quality assessment results of the included studies. All included studies compared their results to the criterion standard, and items 1, 2, 3, 5, 6, 7, 8, 9, 10, and 12 were rated "yes," indicating that these studies used the same reference standard as the criterion standard, thereby controlling for bias. All studies received

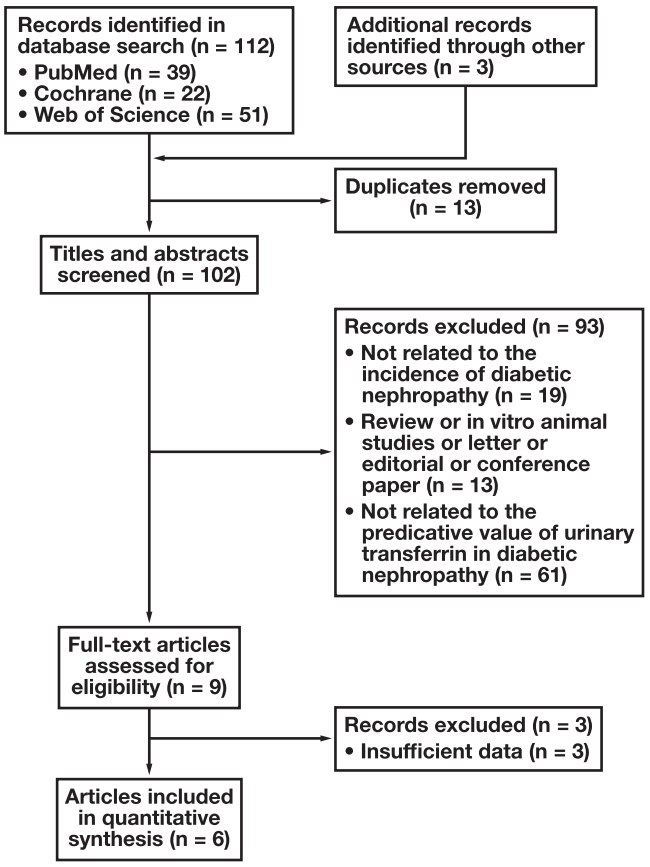
a "no" rating for item 11. Items 13 and 14 were rated "no," indicating a high probability of bias. The judgment results for item 4 were all "unclear," indicating that the time interval between the detection of Tf in the included studies and the detection of the criterion-standard experiment was unclear. This ambiguity suggests that changes in the disease state may have occurred during this period, potentially introducing bias and influencing the interpretation of the results somehow.

Heterogeneity Test

The threshold value of urinary Tf varied among the studies included in this meta-analysis, and some studies did not report the specific threshold value. This disparity in threshold values may contribute to heterogeneity in the magnitude of threshold-induced effects. To remedy this, we performed a threshold effect test. Using Meta-DiSc version 1.4, a ROC plane scatterplot of urine Tf was generated, which revealed the absence of "shoulder-like" variations. Also, the Spearman correlation coefficient was determined to be 0.417 (P =.54). These results prove that no substantial threshold effect has been observed across the included studies.

In this study, the Cochran-Q test for the DOR revealed a Cochran-Q value of 48.91 (P < .05), indicating the presence of heterogeneity due to nonthreshold effects. The subsequent analysis revealed that the sensitivity, specificity, and I^2 values of the PLR, the NLR, and the DOR were all >50%. Therefore, the present study employed a random-effects model to combine these 5 effect sizes.

FIGURE 1. Study selection flowchart.



Pooling of Effect Sizes for Diagnostic Value Assessment

Based on the included literature, a meta-analysis was conducted to combine the effect sizes of urinary Tf for the diagnostic value in DN. The diagnostic value of urinary Tf in DN was discussed in all 6 articles included in this study. **TABLE 3** contains a summary of the research findings. The pooled sensitivity was 0.82 (95% CI, 0.71-0.89), the specificity was 0.88 (0.84-0.92), PLR was 7.07 (4.57-10.93), NLR was 0.20 (0.12-0.35), DOR was 34.49 (13.61-87.44), and the AUC of the summary ROC was 0.92 (0.89-0.94) (Supplemental Figures 1-4).

Publication Bias Analysis

The results of Deeks funnel plot analysis of the 6 studies included in the meta-analysis demonstrated an even distribution of DOR values on both sides of the pooled effect size (P = .77). These findings indicate the absence of significant publication bias among the included studies in the meta-analysis (**FIGURE 2A**).

Evaluation of Clinical Effect of Urine Tf in the Diagnosis of DN

Using the Fagan nomogram, the clinical utility of urinary Tf in diagnosing DN was evaluated. Using a pretest probability of 50%, the posttest probability would be 88%. This finding indicated that the risk of developing DN increased from 50% to 88% in patients with diabetes whose urinary Tf levels exceeded the threshold. In contrast, when the pretest probability was set to 50%, the posttest probability decreased to 17% when the urinary Tf levels of the patient with diabetes were below the threshold. These results confirmed the diagnostic significance of urinary Tf in DN (**FIGURE 2B**).

TABLE 1. Baseline Characteristics of Included Studies for Meta-Analysis

First author, year	Country	No. of cases		DN grouping	TP	FP	FN	TN	Cut-off value	Staging criteria for DN	Test method
		DN	DM								
Żyłka, 2018[20]	Poland	19	61	NO/MI	14	4	5	57	3.59 g/L	UACR	Immunonephelometric assay
Terzić, 2019[21]	Serbia	33	47	NO/MI	27	9	6	38	NA	UACR	ELISA
Kamel, 2022[22]	Egypt	40	20	NO/MI/MA	34	3	6	17	0.85 ng/mL	UACR	ELISA
Zhang, 2019[23]	China	143	144	NO/MI/MA	100	18	43	126	3.49 mg/L	UACR	Immunonephelometric assay
Al-Rubeaan, 2017[24]	Saudi Arabia	267	200	NO/MI/MA	187	34	80	166	NA	UACR	ELISA
Hu, 2019[25]	China	200	100	NO/MI/MA	190	6	10	94	NA	UACR	ELISA

DM, diabetes mellitus; DN, diabetic nephropathy; ELISA, enzyme-linked immunosorbent assay; FN, false negative; FP, false positive; MA, macroalbuminuria; MI, microalbuminuria; NA, nonapplicable; NO, normal albuminuria; TN, true negative; TP, true positive; UACR, urine albumin-to-creatinine ratio.

TABLE 2. Details of Quality Assessment With QUADAS-2 Scale Diagnostic Quality Evaluation Form

First author, year	Patient selection				Index test		Reference standard			Flow and timing			
	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Q13	Q14
Żyłka, 2018[20]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk
Terzić, 2019[21]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk
Kamel, 2022[22]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk
Zhang, 2019[23]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk
Al-Rubeaan, 2017[24]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk
Hu, 2019[25]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk

Discussion

DN is the primary cause of ESKD and kidney replacement therapy in economically developed nations. In patients with diabetes, early detection of kidney impairment is crucial for effectively delaying its progression and improving quality of life. Typically, microalbuminuria and a declining GFR are used to diagnose DN.[26] However, microalbuminuria by itself may not be a sensitive and specific predictor of DN because only 30% of individuals with microalbuminuria develop clinical nephropathy after 10 years of follow-up.[27] Also, 30%-45% of patients with type 2 diabetes (T2D) and DN have decreased eGFR without an increase in UACR—a condition known as normoalbuminuric diabetic nephropathy. In contrast, early kidney impairment in patients with diabetes frequently manifests as glomerular hyperfiltration, with later eGFR reductions.[28] An optimal diagnostic marker for DN must possess multiple qualities. First, it must demonstrate a strong correlation between the degree of proteinuria and the progression of kidney function decline. Second, as DN progresses, the marker levels should increase from microalbuminuria to macroalbuminuria. Finally, the marker should have an early prognostic influence on kidney function impairment in patients with diabetes who have normal albuminuria.[28]

Urinary Tf levels may be a more sensitive marker than microalbuminuria, the current criterion standard, for detecting glomerular impairment and nephropathy in patients with diabetes.[29,30] Study results[15,31] have shown a significant correlation between urinary Tf excretion and urinary microalbumin excretion in patients with T2D, with urinary Tf levels increasing concurrently with urinary microalbumin levels and exceeding the magnitude of increase observed in urinary microalbumin by itself.[15,32]

We note that 2 studies[15,33] reported observing elevated urinary Tf levels in more than 30% of patients with T2D who had normal proteinuria. Zhang et al[23] discovered that 61% of patients with normoalbuminuria, 95% of patients with microalbuminuria, and 100% of patients with macroalbuminuria with T2D had aberrant urinary Tf/creatinine ratios. Increased urinary Tf excretion was associated with an increased risk of developing microalbuminuria in individuals with normoalbuminurias.[30,34] Also, Kazumi et al [34] reported that 31% of patients with T2D and elevated urinary Tf levels developed microalbuminuria, compared with only 7% of patients testing Tf negative. These results demonstrated the potential value of urine Tf testing in patients with diabetes and microalbuminuria, as well as patients with diabetes and normoalbuminuria, highlighting the potential of Tf as a more sensitive marker for detecting glomerular dysfunction than microalbuminuria.[35]

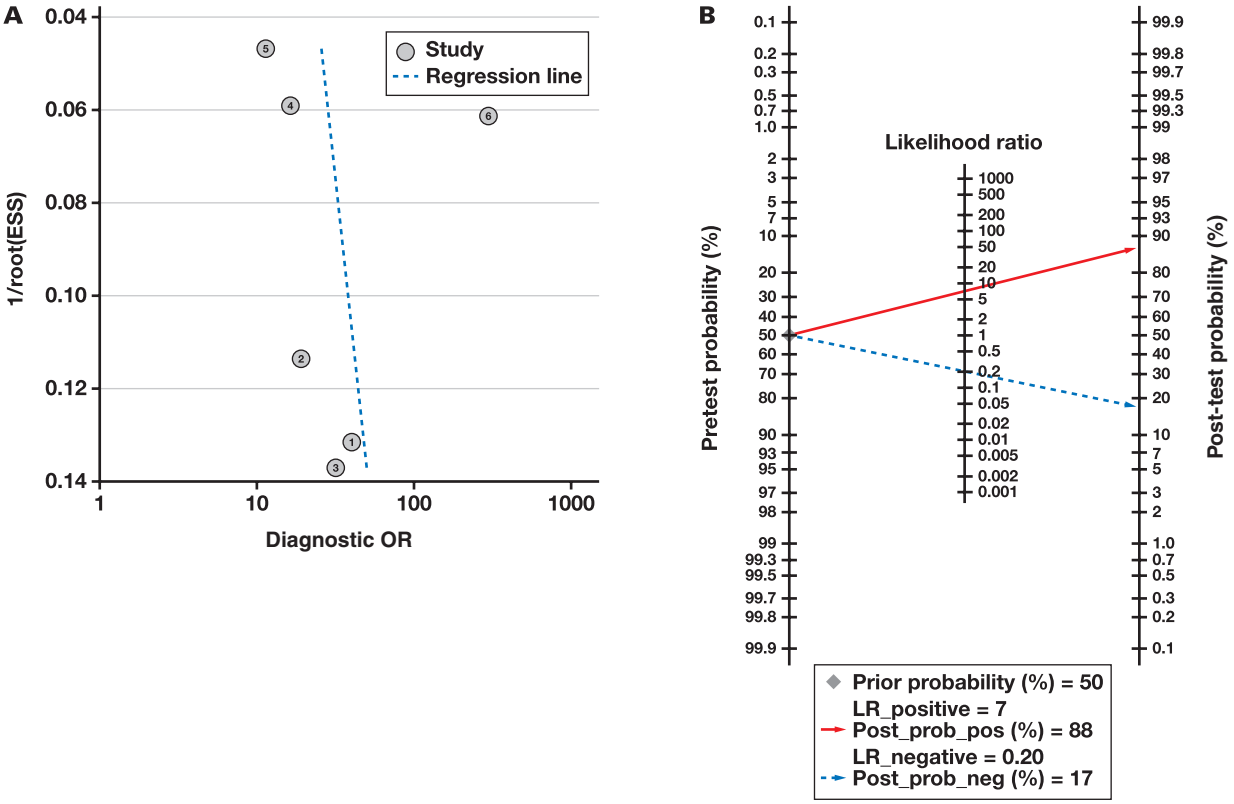
Urinary Tf is a glomerular marker that increases considerably with the progression of diffuse glomerular disease and maintains a close association with renal function impairment. Due to structural differences, Tf is typically less anionic than albumin, and its isoelectric point is approximately 1 unit higher. Thus, urinary Tf may be excreted in the early phases of DN before microalbumin. Tf readily traverses the glomerular barrier alongside albumin during late-stage renal injury, and increased Tf excretion indicates decreased tubular reabsorption.[36] In the results of a study by Siddiqui et al,[37] urinary Tf levels increased 5-fold in the moderately increased risk group, whereas increased albumin excretion and decreased eGFR did not extend to the progressive kidney disease stage. Based on microalbumin classification, urinary Tf increases in tandem with albumin excretion, indicating that glomerular permeability changes predominantly influence its excretion.[37]

TABLE 3. Pooled Effect Size for Diagnostic Value Assessment

First author, year	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)	DOR (95% CI)
Żyłka, 2018[20]	0.74 (0.49-0.91)	0.93 (0.84-0.98)	11.24 (4.20-30.08)	0.28 (0.13-0.60)	39.90 (9.46-168.22)
Terzić, 2019[21]	0.82 (0.65-0.93)	0.81 (0.67-0.91)	4.27 (2.32-7.86)	0.22 (0.11-0.47)	19.00 (6.05-59.69)
Kamel, 2022[22]	0.85 (0.70-0.94)	0.85 (0.62-0.97)	5.67 (1.98-16.22)	0.18 (0.08-0.38)	32.11 (7.14-144.38)
Zhang, 2019[23]	0.70 (0.62-0.77)	0.88 (0.81-0.92)	5.59 (3.58-8.73)	0.34 (0.27-0.44)	16.28 (8.85-29.95)
Al-Rubeaan, 2017[24]	0.70 (0.64-0.75)	0.83 (0.77-0.88)	4.12 (3.00-5.65)	0.36 (0.30-0.44)	11.41 (7.26-17.94)
Hu, 2019[25]	0.95 (0.91-0.98)	0.94 (0.87-0.98)	15.83 (7.28-34.42)	0.05 (0.03-0.10)	297.67 (105.02-843.73)
Pooled effect size	0.82 (0.71-0.89)	0.88 (0.84-0.92)	7.07 (4.57-10.93)	0.20 (0.12-0.35)	34.49 (13.61-87.44)

DOR, diagnostic odds ratio; NLR, negative diagnostic likelihood ratio; PLR, positive diagnostic likelihood ratio.

FIGURE 2. A, Deeks funnel plot for publication assessment (*P* = .77). B, Fagan nomogram in detecting diagnostic probability of urinary transferrin for diabetic nephropathy. LR, likelihood ratio; neg, negativity; pos, positivity; prob, probability.



Kanauchi et al[15] found in a previous study that urinary Tf levels increased as renal injury progressed and demonstrated a significant correlation with urinary microalbumin. However, the correlation between Tf and eGFR was no longer significant after adjusting for albumin creatinine ratio. Jiang et al[38] evaluated T2D nephropathy in their study by examining the relationship between urine markers, eGFR, and UAER. The results revealed elevated urinary Tf levels in all groups, which correlated negatively with eGFR in patients with diabetes and positively with UAER. Also, the study identified elevated urinary Tf levels in patients with diabetes who had normal eGFR, indicating that urinary Tf could serve as a sensitive marker for the early detection of diabetic kidney injury.[38]

Urinary Tf levels increase gradually and significantly as DN progresses. In a study by Kazumi et al,[34] 95% of patients with microalbuminuria and 100% of patients with macroalbuminuria had elevated urinary transferrin excretion. Narita et al[30] found in a 5-year follow-up study that elevated urinary Tf levels could be used to predict the progression of microalbuminuria in patients with T2D. These results support the theory that urinary Tf is a sensitive and specific diagnostic marker for DN.

We included 6 studies in our meta-analysis of the diagnostic value of urinary Tf in early DN. In predicting DN, urine Tf demonstrated a combined sensitivity and specificity of 0.82 and 0.88, respectively. Higher PLRs indicate a more considerable diagnostic value, whereas lower NLRs indicate lower disease likelihood. However, in clinical practice, several factors can result in false-negative or false-positive test results for specific diseases. PLR >5 or NLR <0.2 typically suggests a marker or detection method with a high diagnostic value.[39] Therefore, it may be more appropriate to interpret the results based on the PLR.

In our analysis, the pooled PLR for patients with diabetes who had elevated urinary transferrin was 7.07, indicating a high probability of

diagnosing DN. The pooled NLR was 0.20, indicating that patients with diabetes who had normal urine Tf values had a reduced risk of developing DN. Also, the summary ROC curve provides a comprehensive evaluation of the value of a diagnostic test. Our study revealed a summary ROC AUC of 0.92, indicating that urinary Tf has a high predictive value for DN.

The DOR represents diagnostic performance, with greater values indicating superior performance. Our aggregated study yielded a DOR of 34.49, providing additional support for the diagnostic utility of urinary Tf for DN. When evaluating the clinical value of urinary Tf in the diagnosis of DN using the Fagan nomogram, the results confirmed the clinical significance of urine Tf.

This study has several limitations. First, the diagnostic value of urinary Tf for DN was evaluated solely based on UACR or UAER without considering the relationship between Tf levels and alterations in kidney function in patients with diabetes. Also, the included studies were cross-sectional, emphasizing the need for additional prospective studies to investigate the predictive value of Tf in the progressive decline of kidney function. Further, our study sample size was comparatively small, necessitating further research to validate the diagnosis and utility of urinary Tf in DN. Future research should also investigate the integration of urinary Tf with other clinical indicators to improve the diagnostic accuracy of DN.

Conclusion

Tf level is a valuable biological marker for early diagnosing and monitoring DN in clinical practice. It has a high predictive value for patients in the initial stages of DN.

Supplementary Material

Supplementary material is available at *Laboratory Medicine* online.

Acknowledgments

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

Conflict of Interest Disclosure

The authors have nothing to disclose.

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OVERVIEW

A meta-analysis of urinary transferrin for early diagnosis of diabetic nephropathy

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Key words: diabetic kidney disease; urine; transferrin; diagnostic value; meta-analysis; early diagnosis

Abbreviations: CKD, chronic kidney disease; DN, diabetic nephropathy;ESRD, end-stage renal disease; GFR, glomerular filtration rate; eGFR, estimated glomerular filtration rate; Tf, transferrin; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; UAER, urinary microalbumin excretion rate; UACR, urinary microalbumin/creatinine ratio; TP, true positive; FP, false positive; FN, false negative; TN, true negative; PLR, positive diagnostic likelihood ratio; NLR, negative diagnostic likelihood ratio; DOR, diagnostic odds ratio; T2D, type 2 diabetes; ROC, receiver operating characteristic; LR, likelihood ratio; prob, probability; pos, positivity; neg, negativity;DM, diabetes mellitus;NO, normal albuminuria;MI, microalbuminuria; MA, macroalbuminuria; NA nonapplicable

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ABSTRACT

Objective: To assess the diagnostic value of urinary transferrin (Tf) in early diabetic nephropathy (DN) to propose a more sensitive and noninvasive biomarker for screening and monitoring DN in clinical practice.

Methods: We searched 3 databases from their inception to May 2023, to identify studies investigating the diagnostic value of Tf in patients with DN. Meta-DiSc software, version 1.4, and Stata software, version 15.1 (StataCorp) were used to conduct a meta-analysis and evaluate the diagnostic accuracy of urine Tf levels for DN.

Results: The meta-analysis included 6 relevant studies investigating the diagnostic value of Tf level for DN. Urinary Tf as a diagnostic marker demonstrated a combined sensitivity of 0.82 (95% CI, 0.71-0.89) and specificity of 0.88 (0.84-0.92). the positive diagnostic likelihood ratio was 7.07 (4.57-10.93), the negative diagnostic likelihood ratio was 0.20 (0.12-0.35), and the diagnostic odds ratio was 34.49 (13.61-87.44). Also, the area under the receiver operating characteristic curve was 0.92 (0.89-0.94), indicating that urinary Tf has a decent discriminative ability in diagnosing DN.

Conclusion: Tf level is a valuable biological marker for early diagnosis and monitoring of DN in clinical practice. It has statistically significant predictive value for patients in the early phases of DN.

Introduction

Patients with diabetes are approximately twice as likely to develop chronic kidney disease (CKD) as those without diabetes.[1] Diabetic nephropathy (DN) is characterized predominantly by persistent proteinuria, progressive decline in renal function, and elevated serum creatinine levels. Inadequate or ineffectual treatment may lead to the development of chronic renal failure, acute renal failure, end-stage renal disease (ESRD), and various nephrotic syndromes, which may result in death due to the protracted course of the disease.[2]

Microalbuminuria serves as an early diagnostic indicator of DN.[3] However, extensive research findings have demonstrated the rising incidence of nonproteinuric CKD (estimated glomerular filtration rate [eGFR] ≤60 mL/min/1.73 m^2 without proteinuria) among patients with diabetes.[2-4] Therefore, early detection of these patients with nonproteinuric CKD among the patient population with diabetes is crucial.

Blood urea nitrogen (BUN), creatinine, eGFR, and proteinuria are currently the leading indicators for clinical diagnosis of DN and severity assessment.[5,6] However, to a certain extent, these markers are not optimal for assessing early DN. The findings of another study[7] revealed that 20%-40% of patients with diabetes experienced a decline in GFR before the detection of albuminuria. In this 10-year follow-up study, Fioretto et al[7] found that 30%-45% of patients with microalbuminuria progressed to having macroalbuminuria. In total, 30% of patients returned to normal levels of urine protein, and 30%-40% of patients continued to have microalbuminuria.[7]

In clinical practice, the albumin-to-creatinine ratio is the most common urinary biomarker for diagnosing patients who have diabetes with co-occurring renal disease. Also, eGFR is a crucial indicator for evaluating kidney function and determining the stages of kidney disease. Both markers are important tools for patient management because they provide an estimation of kidney function, which helps in determining the CKD stage and in guiding therapy.[8] However, the sensitivity of these markers in detecting subtle changes in renal function is limited.[9] Although renal biopsy remains the criterion standard for

diagnosing DN, its invasiveness limits its use in routine clinical practice. Therefore, it is necessary to investigate more sensitive and noninvasive markers in this discipline.

DN predominantly affects the glomeruli, renal tubules, and renal interstitium with pathological changes. Glomerular injury causes an increase in the urinary excretion of plasma proteins,[10] such as urinary transferrin (Tf), urinary ceruloplasmin, and urinary immunoglobulin G, which serve as markers of glomerular injury. The proportional increase in urinary Tf, urinary immunoglobulin, and urinary ceruloplasmin levels suggests their potential as early biomarkers for DN.[11] Early-stage DN is characterized by increased glomerular permeability, which results in albumin and other plasma proteins (such as Tf) leaking into the urine from the plasma.

Despite having a comparable molecular weight to albumin, Tf has a higher isoelectric point.[12] Due to alterations in its charge, the charge barrier of the glomerular basement membrane weakens in the early phases of DN. Consequently, Tf encounters diminished repulsive forces, facilitating its leakage and providing a more accurate reflection of charge barrier damage.[12] Injuries to the renal tubules are another vital characteristic of DN.[13] Before the advent of microalbuminuria, markers of tubular damage might have been elevated in patients with diabetes.[14] Kanauchi et al[15] reported a positive correlation between tubular dysfunction and urinary levels of microalbumin and Tf in patients with DN.

Hence, the objective of this study is to conduct a meta-analysis to assess the screening efficacy of urinary Tf in early DN. Our aim is to propose a highly sensitive and noninvasive biomarker for the screening and monitoring of DN in clinical practice.

Methods

This meta-analysis was performed according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[16]

Retrieval Strategy

Using predefined keyword search terms, we conducted a comprehensive literature search in PubMed, Cochrane Library, and Web of Science. The search terms used included "diabetic nephropathy," "diabetic kidney disease," "DKD," "DN," "transferrin," and "TRF." To accommodate the specific requirements of each database, the search strategy was refined using a combination of subject terms and free terms. The literature search was conducted from the inception of the databases until May 2023, with no language restrictions.

Literature Inclusion and Exclusion

The study inclusion criteria were defined as follows: adult participants (aged ≥18 years) with diabetes mellitus, DN staging based on urinary microalbumin excretion rate (UAER) or urinary microalbumin/creatinine ratio (UACR), and investigation of the predictive value of urinary Tf in DN using receiver operating characteristic (ROC) curve analysis. There were no restrictions on language, publication year, or publication type. The exclusion criteria were as follows: comments, letters, or conference abstracts; studies lacking true positive (TP), false positive (FP), false negative (FN), and true negative (TN) results; participants with severe infection, diabetic ketoacidosis, primary kidney disease, malignant tumors, or drug-induced renal injury; and duplicate publications.

Data Extraction and Literature Quality Assessment

According to the inclusion and exclusion criteria, 2 independent researchers extracted the following data from each included study: first author; publication year; study location; sample size; standards and classification of DN; method of urinary Tf detection; Tf threshold; number of TP, FP, TN, and FN occurrences; and cutoff value of Tf. Group discussions involving a third researcher resolved any disagreements between the 2 researchers. The quality of the included studies was evaluated using the QUADAS-2 scale,[17] which consists of 14 evaluation items, each with 3 response options (yes, no, or unclear). The 2 evaluators evaluated each study independently, line by line, and discussed any disagreements with the third researcher.

Statistical Analysis

Meta-Disc software, version 1.4, and Stata software, version 15.1, were utilized in this investigation for diagnostic meta-analysis.[18,19] Several indicators, including sensitivity, specificity, positive diagnostic likelihood ratio (PLR), negative diagnostic likelihood ratio (NLR), diagnostic odds ratio (DOR), and area under the curve (AUC), were used to evaluate the diagnostic value of urinary Tf for DN.

First, to assess heterogeneity among the included studies, we used Meta-Disc, version 1.4, employing an ROC scatterplot and Spearman correlation coefficient method. Next, using Stata version 15.1, the effect size, sensitivity, specificity, PLR, NLR, DOR, AUC, and their respective 95% CI values were subsequently analyzed. If there was heterogeneity between the studies ($P < .05$ or $I^2 > 50\%$), a random-effects model was used; otherwise, a fixed-effects model was used. Finally, publication bias was assessed via Deeks funnel plotting; $P < .05$ was considered statistically significant.

Results

Literature Screening, Basic Characteristics, and Quality Evaluation

We initially retrieved 115 articles from the databases mentioned herein. Ultimately, 6 studies were included in the meta-analysis after screening (**FIGURE 1**).[20-25] These 6 cross-sectional studies, involving 1274 participants, were published between 2017 and 2022. Two of the papers were limited to the microalbuminuria group, and the remaining 4 included the microalbuminuria and macroalbuminuria groups. The enzyme-linked immunosorbent assay or the immunoturbidimetric method was used to detect Tf in urine. DN was diagnosed based on UAER or UACR criteria. For data extraction, pertinent information was recorded, including the first author; publication year; country; total sample size; number of cases in the DN group; number of control individuals; number of TP, FP, FN, and TN occurrences; DN diagnosis; Tf threshold; and urine Tf detection method (**TABLE 1**).[20-25]

We evaluated the 6 articles included in this study using the QUADAS-2 scale diagnostic quality evaluation form. There are a total of 14 items on the scale. Each item was assessed based on its applicability to the included studies, using the 3 response options "yes," "no," and "unclear." **TABLE 2** displays the quality assessment results of the included studies. All included studies compared their results to the criterion standard, and items 1, 2, 3, 5, 6, 7, 8, 9, 10, and 12 were rated "yes," indicating that these studies used the same reference standard as the criterion standard, thereby controlling for bias. All studies received

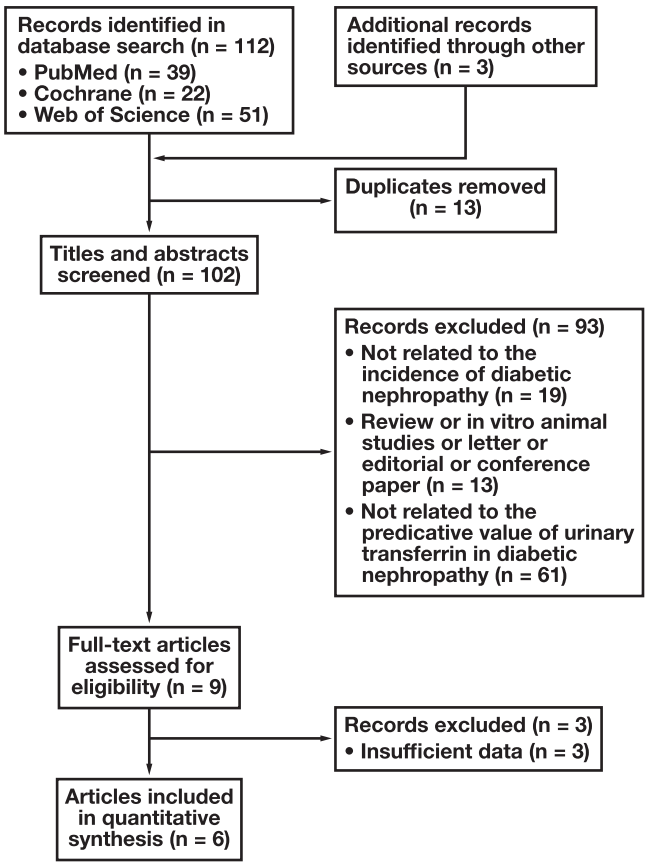
a "no" rating for item 11. Items 13 and 14 were rated "no," indicating a high probability of bias. The judgment results for item 4 were all "unclear," indicating that the time interval between the detection of Tf in the included studies and the detection of the criterion-standard experiment was unclear. This ambiguity suggests that changes in the disease state may have occurred during this period, potentially introducing bias and influencing the interpretation of the results somehow.

Heterogeneity Test

The threshold value of urinary Tf varied among the studies included in this meta-analysis, and some studies did not report the specific threshold value. This disparity in threshold values may contribute to heterogeneity in the magnitude of threshold-induced effects. To remedy this, we performed a threshold effect test. Using Meta-DiSc version 1.4, a ROC plane scatterplot of urine Tf was generated, which revealed the absence of "shoulder-like" variations. Also, the Spearman correlation coefficient was determined to be 0.417 (P =.54). These results prove that no substantial threshold effect has been observed across the included studies.

In this study, the Cochran-Q test for the DOR revealed a Cochran-Q value of 48.91 (P < .05), indicating the presence of heterogeneity due to nonthreshold effects. The subsequent analysis revealed that the sensitivity, specificity, and I^2 values of the PLR, the NLR, and the DOR were all >50%. Therefore, the present study employed a random-effects model to combine these 5 effect sizes.

FIGURE 1. Study selection flowchart.



Pooling of Effect Sizes for Diagnostic Value Assessment

Based on the included literature, a meta-analysis was conducted to combine the effect sizes of urinary Tf for the diagnostic value in DN. The diagnostic value of urinary Tf in DN was discussed in all 6 articles included in this study. **TABLE 3** contains a summary of the research findings. The pooled sensitivity was 0.82 (95% CI, 0.71-0.89), the specificity was 0.88 (0.84-0.92), PLR was 7.07 (4.57-10.93), NLR was 0.20 (0.12-0.35), DOR was 34.49 (13.61-87.44), and the AUC of the summary ROC was 0.92 (0.89-0.94) (Supplemental Figures 1-4).

Publication Bias Analysis

The results of Deeks funnel plot analysis of the 6 studies included in the meta-analysis demonstrated an even distribution of DOR values on both sides of the pooled effect size (P = .77). These findings indicate the absence of significant publication bias among the included studies in the meta-analysis (**FIGURE 2A**).

Evaluation of Clinical Effect of Urine Tf in the Diagnosis of DN

Using the Fagan nomogram, the clinical utility of urinary Tf in diagnosing DN was evaluated. Using a pretest probability of 50%, the posttest probability would be 88%. This finding indicated that the risk of developing DN increased from 50% to 88% in patients with diabetes whose urinary Tf levels exceeded the threshold. In contrast, when the pretest probability was set to 50%, the posttest probability decreased to 17% when the urinary Tf levels of the patient with diabetes were below the threshold. These results confirmed the diagnostic significance of urinary Tf in DN (**FIGURE 2B**).

TABLE 1. Baseline Characteristics of Included Studies for Meta-Analysis

First author, year	Country	No. of cases		DN grouping	TP	FP	FN	TN	Cut-off value	Staging criteria for DN	Test method
		DN	DM								
Żyłka, 2018[20]	Poland	19	61	NO/MI	14	4	5	57	3.59 g/L	UACR	Immunonephelometric assay
Terzić, 2019[21]	Serbia	33	47	NO/MI	27	9	6	38	NA	UACR	ELISA
Kamel, 2022[22]	Egypt	40	20	NO/MI/MA	34	3	6	17	0.85 ng/mL	UACR	ELISA
Zhang, 2019[23]	China	143	144	NO/MI/MA	100	18	43	126	3.49 mg/L	UACR	Immunonephelometric assay
Al-Rubeaan, 2017[24]	Saudi Arabia	267	200	NO/MI/MA	187	34	80	166	NA	UACR	ELISA
Hu, 2019[25]	China	200	100	NO/MI/MA	190	6	10	94	NA	UACR	ELISA

DM, diabetes mellitus; DN, diabetic nephropathy; ELISA, enzyme-linked immunosorbent assay; FN, false negative; FP, false positive; MA, macroalbuminuria; MI, microalbuminuria; NA, nonapplicable; NO, normal albuminuria; TN, true negative; TP, true positive; UACR, urine albumin-to-creatinine ratio.

TABLE 2. Details of Quality Assessment With QUADAS-2 Scale Diagnostic Quality Evaluation Form

First author, year	Patient selection				Index test		Reference standard			Flow and timing			
	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Q13	Q14
Żyłka, 2018[20]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk
Terzić, 2019[21]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk
Kamel, 2022[22]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk
Zhang, 2019[23]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk
Al-Rubeaan, 2017[24]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk
Hu, 2019[25]	Yes	Yes	Yes	Unclear risk	Yes	Yes	Low risk	Yes	Yes	Low risk	No	Yes	No	High risk

Discussion

DN is the primary cause of ESKD and kidney replacement therapy in economically developed nations. In patients with diabetes, early detection of kidney impairment is crucial for effectively delaying its progression and improving quality of life. Typically, microalbuminuria and a declining GFR are used to diagnose DN.[26] However, microalbuminuria by itself may not be a sensitive and specific predictor of DN because only 30% of individuals with microalbuminuria develop clinical nephropathy after 10 years of follow-up.[27] Also, 30%-45% of patients with type 2 diabetes (T2D) and DN have decreased eGFR without an increase in UACR—a condition known as normoalbuminuric diabetic nephropathy. In contrast, early kidney impairment in patients with diabetes frequently manifests as glomerular hyperfiltration, with later eGFR reductions.[28] An optimal diagnostic marker for DN must possess multiple qualities. First, it must demonstrate a strong correlation between the degree of proteinuria and the progression of kidney function decline. Second, as DN progresses, the marker levels should increase from microalbuminuria to macroalbuminuria. Finally, the marker should have an early prognostic influence on kidney function impairment in patients with diabetes who have normal albuminuria.[28]

Urinary Tf levels may be a more sensitive marker than microalbuminuria, the current criterion standard, for detecting glomerular impairment and nephropathy in patients with diabetes.[29,30] Study results[15,31] have shown a significant correlation between urinary Tf excretion and urinary microalbumin excretion in patients with T2D, with urinary Tf levels increasing concurrently with urinary microalbumin levels and exceeding the magnitude of increase observed in urinary microalbumin by itself.[15,32]

We note that 2 studies[15,33] reported observing elevated urinary Tf levels in more than 30% of patients with T2D who had normal proteinuria. Zhang et al[23] discovered that 61% of patients with normoalbuminuria, 95% of patients with microalbuminuria, and 100% of patients with macroalbuminuria with T2D had aberrant urinary Tf/creatinine ratios. Increased urinary Tf excretion was associated with an increased risk of developing microalbuminuria in individuals with normoalbuminurias.[30,34] Also, Kazumi et al [34] reported that 31% of patients with T2D and elevated urinary Tf levels developed microalbuminuria, compared with only 7% of patients testing Tf negative. These results demonstrated the potential value of urine Tf testing in patients with diabetes and microalbuminuria, as well as patients with diabetes and normoalbuminuria, highlighting the potential of Tf as a more sensitive marker for detecting glomerular dysfunction than microalbuminuria.[35]

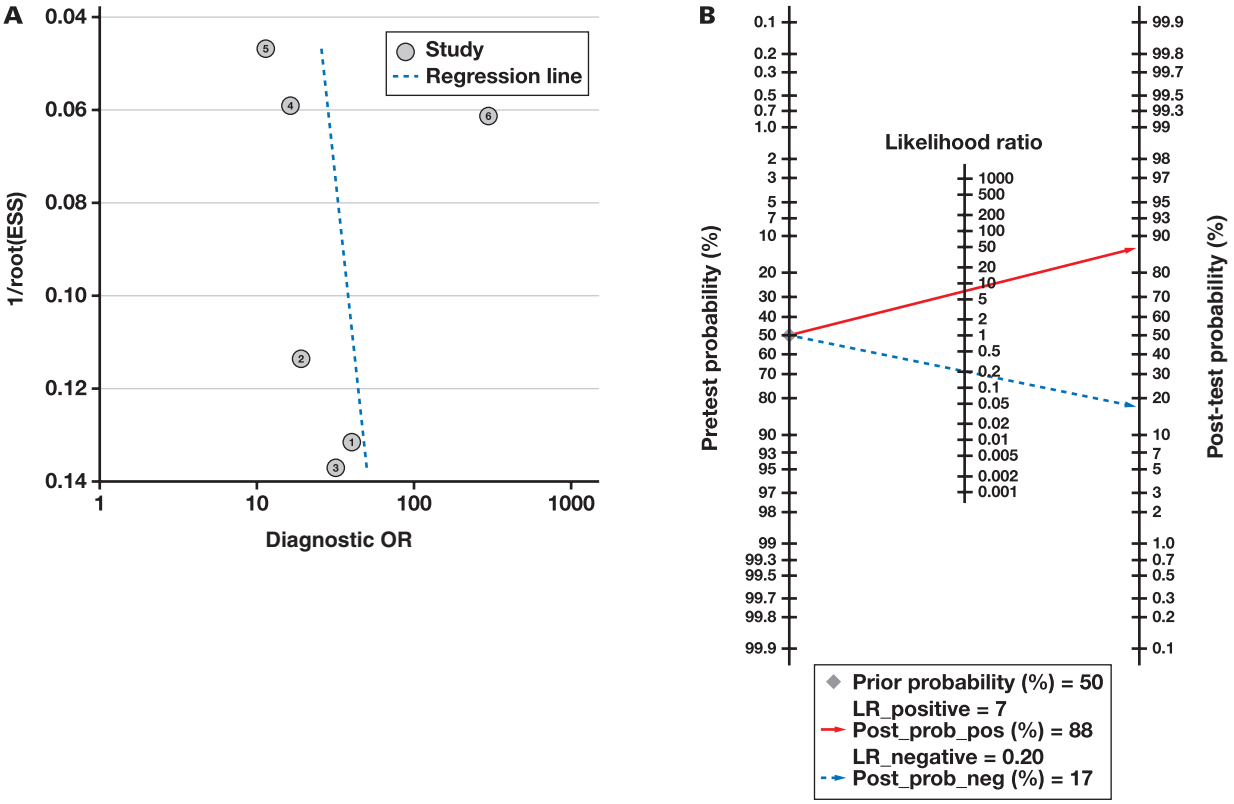
Urinary Tf is a glomerular marker that increases considerably with the progression of diffuse glomerular disease and maintains a close association with renal function impairment. Due to structural differences, Tf is typically less anionic than albumin, and its isoelectric point is approximately 1 unit higher. Thus, urinary Tf may be excreted in the early phases of DN before microalbumin. Tf readily traverses the glomerular barrier alongside albumin during late-stage renal injury, and increased Tf excretion indicates decreased tubular reabsorption.[36] In the results of a study by Siddiqui et al,[37] urinary Tf levels increased 5-fold in the moderately increased risk group, whereas increased albumin excretion and decreased eGFR did not extend to the progressive kidney disease stage. Based on microalbumin classification, urinary Tf increases in tandem with albumin excretion, indicating that glomerular permeability changes predominantly influence its excretion.[37]

TABLE 3. Pooled Effect Size for Diagnostic Value Assessment

First author, year	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)	DOR (95% CI)
Żyłka, 2018[20]	0.74 (0.49-0.91)	0.93 (0.84-0.98)	11.24 (4.20-30.08)	0.28 (0.13-0.60)	39.90 (9.46-168.22)
Terzić, 2019[21]	0.82 (0.65-0.93)	0.81 (0.67-0.91)	4.27 (2.32-7.86)	0.22 (0.11-0.47)	19.00 (6.05-59.69)
Kamel, 2022[22]	0.85 (0.70-0.94)	0.85 (0.62-0.97)	5.67 (1.98-16.22)	0.18 (0.08-0.38)	32.11 (7.14-144.38)
Zhang, 2019[23]	0.70 (0.62-0.77)	0.88 (0.81-0.92)	5.59 (3.58-8.73)	0.34 (0.27-0.44)	16.28 (8.85-29.95)
Al-Rubeaan, 2017[24]	0.70 (0.64-0.75)	0.83 (0.77-0.88)	4.12 (3.00-5.65)	0.36 (0.30-0.44)	11.41 (7.26-17.94)
Hu, 2019[25]	0.95 (0.91-0.98)	0.94 (0.87-0.98)	15.83 (7.28-34.42)	0.05 (0.03-0.10)	297.67 (105.02-843.73)
Pooled effect size	0.82 (0.71-0.89)	0.88 (0.84-0.92)	7.07 (4.57-10.93)	0.20 (0.12-0.35)	34.49 (13.61-87.44)

DOR, diagnostic odds ratio; NLR, negative diagnostic likelihood ratio; PLR, positive diagnostic likelihood ratio.

FIGURE 2. A, Deeks funnel plot for publication assessment (*P* = .77). B, Fagan nomogram in detecting diagnostic probability of urinary transferrin for diabetic nephropathy. LR, likelihood ratio; neg, negativity; pos, positivity; prob, probability.



Kanauchi et al[15] found in a previous study that urinary Tf levels increased as renal injury progressed and demonstrated a significant correlation with urinary microalbumin. However, the correlation between Tf and eGFR was no longer significant after adjusting for albumin creatinine ratio. Jiang et al[38] evaluated T2D nephropathy in their study by examining the relationship between urine markers, eGFR, and UAER. The results revealed elevated urinary Tf levels in all groups, which correlated negatively with eGFR in patients with diabetes and positively with UAER. Also, the study identified elevated urinary Tf levels in patients with diabetes who had normal eGFR, indicating that urinary Tf could serve as a sensitive marker for the early detection of diabetic kidney injury.[38]

Urinary Tf levels increase gradually and significantly as DN progresses. In a study by Kazumi et al,[34] 95% of patients with microalbuminuria and 100% of patients with macroalbuminuria had elevated urinary transferrin excretion. Narita et al[30] found in a 5-year follow-up study that elevated urinary Tf levels could be used to predict the progression of microalbuminuria in patients with T2D. These results support the theory that urinary Tf is a sensitive and specific diagnostic marker for DN.

We included 6 studies in our meta-analysis of the diagnostic value of urinary Tf in early DN. In predicting DN, urine Tf demonstrated a combined sensitivity and specificity of 0.82 and 0.88, respectively. Higher PLRs indicate a more considerable diagnostic value, whereas lower NLRs indicate lower disease likelihood. However, in clinical practice, several factors can result in false-negative or false-positive test results for specific diseases. PLR >5 or NLR <0.2 typically suggests a marker or detection method with a high diagnostic value.[39] Therefore, it may be more appropriate to interpret the results based on the PLR.

In our analysis, the pooled PLR for patients with diabetes who had elevated urinary transferrin was 7.07, indicating a high probability of

diagnosing DN. The pooled NLR was 0.20, indicating that patients with diabetes who had normal urine Tf values had a reduced risk of developing DN. Also, the summary ROC curve provides a comprehensive evaluation of the value of a diagnostic test. Our study revealed a summary ROC AUC of 0.92, indicating that urinary Tf has a high predictive value for DN.

The DOR represents diagnostic performance, with greater values indicating superior performance. Our aggregated study yielded a DOR of 34.49, providing additional support for the diagnostic utility of urinary Tf for DN. When evaluating the clinical value of urinary Tf in the diagnosis of DN using the Fagan nomogram, the results confirmed the clinical significance of urine Tf.

This study has several limitations. First, the diagnostic value of urinary Tf for DN was evaluated solely based on UACR or UAER without considering the relationship between Tf levels and alterations in kidney function in patients with diabetes. Also, the included studies were cross-sectional, emphasizing the need for additional prospective studies to investigate the predictive value of Tf in the progressive decline of kidney function. Further, our study sample size was comparatively small, necessitating further research to validate the diagnosis and utility of urinary Tf in DN. Future research should also investigate the integration of urinary Tf with other clinical indicators to improve the diagnostic accuracy of DN.

Conclusion

Tf level is a valuable biological marker for early diagnosing and monitoring DN in clinical practice. It has a high predictive value for patients in the initial stages of DN.

Supplementary Material

Supplementary material is available at *Laboratory Medicine* online.

Acknowledgments

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

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Diagnostic value of plasma circular RNA based on droplet digital polymerase chain reaction in lung adenocarcinoma

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Key words: lung adenocarcinoma, circular RNA, diagnostic markers, droplet digital PCR

Abbreviations: circRNA, circular RNA; PCR, polymerase chain reaction; ddPCR, droplet digital PCR; ROC, receiver operating characteristic; miRNAs, microRNAs; mRNAs, messenger RNAs; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; AUC, area under the ROC curve; FC, fold change; nt, nucleotides

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ABSTRACT

Background: Plasma circular (circ)RNAs detected by droplet digital polymerase chain reaction (ddPCR) may be ideal markers for liquid biopsy. However, ddPCR detection of circRNAs in plasma for diagnosis of lung adenocarcinoma has been rarely reported.

Methods: An RNA sequencing analysis was performed in plasma from patients with early lung adenocarcinoma and healthy individuals. Droplet digital PCR was used to verify the differentially expressed genes.

Results: The copy numbers of circle RNA*LZIC* (circ*LZIC*)and circle RNA*CEP350* (circ*CEP350*) in the plasma of lung adenocarcinoma patients were significantly higher than in plasma of healthy people, and the copy numbers in postoperative plasma of the same patients were significantly lower than those in preoperative plasma. Circ*LZIC* and circ*CEP350* alone and in combination had diagnostic value in lung adenocarcinoma and early lung adenocarcinoma. Circ*LZIC* and circ*CEP350* had more binding sites with multiple microRNAs. Their target genes were enriched in several signaling pathways.

Conclusion: The copy numbers of circ*LZIC* and circ*CEP350* were higher in plasma of lung adenocarcinoma patients than in plasma of healthy controls, significantly correlated with tumor size and TNM stage, and closely related to the occurrence and development of tumors. These circRNAs may serve as molecular markers for the diagnosis of lung adenocarcinoma.

Introduction

Lung cancer has a high morbidity and mortality and represents 40% of non–small cell lung cancer.[1-3] Despite recent advancements in the development of targeted treatment and immunotherapy, clinical prognosis of lung cancer remains poor, with a 20% 5-year survival rate in patients diagnosed with advanced metastatic.[1] Poor outcomes are mainly attributed to ineffective early screening and diagnosis. For example, low-dose computed tomography screening for lung cancer not only exposes patients to radiation, but it also has a high false-positive rate.[4,5] Furthermore, tumor markers such as carcinoembryonic antigen and cytokeratin 19 fragment have low sensitivity and specificity.[6,7] Molecular biology may identify biomarkers with better diagnostic efficacy for the early diagnosis of lung cancer to provide timely diagnosis and improved outcomes. Circular RNA (circRNA), noncoding RNAs with a covalently closed circular structure, are ubiquitously distributed, structurally diverse, and demonstrate a variety of regulatory functions.[8] They play important roles in cancer onset, progression, drug resistance, and therapeutic outcomes.[9] Unlike linear RNA, circRNAs have covalently closed loop structure lakes with a 5′ cap or a 3′ poly A tail.[10] This feature makes circRNA more stable than linear RNA in vivo.[11,12] CircRNA also demonstrates richness, evolutionary conservation, and stability,[13] resulting in them being readily detected in plasma, saliva, and urine. Another important molecular characteristic of circRNAs is their variable expression in patients presenting at different stages of the same cancer.[14-16] Because of these unique features, circRNA may serve as an ideal marker for liquid biopsy.[17]

Due to its minimal invasive nature and ease of collecting multiple samples, liquid biopsy technology is an emerging technique/tool in the early screening of diseases. Because of its stability, circRNA in body fluids

has become a candidate marker for tumor diagnosis.[18] False-negative results from traditional quantitative real-time polymerase chain reaction (qRT-PCR) technology in plasma is due to low abundance of circRNA.[19] Therefore, droplet digital PCR (ddPCR) is more suitable for the detection of circRNA in plasma due to its high sensitivity and precise quantitative detection.[20] However, ddPCR detection of circRNA in plasma for early diagnosis of lung adenocarcinoma has been infrequently reported.

This study demonstrates how RNA sequencing technology can be used to screen differentially expressed circRNAs in the plasma of patients with early lung adenocarcinoma. We used ddPCR to detect the copy number of plasma circRNAs in patients with early lung adenocarcinoma, patients with advanced lung adenocarcinoma, and healthy controls to provide an experimental basis for the identification of accurate molecular markers for lung adenocarcinoma diagnosis. We used receiver operating characteristic (ROC) curve analysis to demonstrate the diagnostic value of combined detection.

Methods

Patient Population

The 55 lung cancer patients included in this study were admitted to the Department of Respiratory and Critical Care Medicine and the Department of Thoracic Surgery in The First Affiliated Hospital of Bengbu Medical College. Patients were diagnosed with lung adenocarcinoma. None of the patients received any treatment (including radiotherapy, targeted therapy, and immune therapy) before plasma samples were taken. The control group included 30 healthy people who underwent physical examination in our hospital during the same period. This study was performed in accordance with the amended Declaration of Helsinki (as revised in 2013), following approval of the study by the Ethics Review Committee of The First Affiliated Hospital of Bengbu Medical College (approval No. 2020-112 and No. 2020-121). All participants provided written informed consent.

Total Plasma RNA Extraction and RNA Quality Detection

Blood samples were collected in tubes containing K2EDTA (Becton Dickinson), centrifuged at 3000*g* for 10 min, and stored at –80°C. Total RNA was isolated from 300 μL plasma using TRIzol-LS (Invitrogen), and the purity and concentration of RNA were determined by ultraviolet spectrophotometry (Thermo Fisher Scientific).

RNA Library Construction and Sequencing

The Clontech SMARTer Stranded Total RNA Seq Kit V2 Pico input kit (Takara) was used to construct RNA libraries. After libraries were initially completed, Qubit 3.0 was used to quantify the libraries and Agilenyzer was used to detect the target library range. Libraries were pooled according to effective concentrations and requirements, and pooled libraries were sequenced in PE150 mode.

Total Plasma RNA RT-PCR and ddPCR to Detect circRNA Copy Numbers

Total RNA was reverse transcribed into cDNA using the RNA reverse transcriptase kit (Takara) in a Mastercycler Gradient PCR instrument (Bio-Rad), and ddPCR analysis was performed using a QX200 instrument.

Primer and Probe Design

Probes were modified with an carboxyfluorescein group in forward primers and a Tamra group in reverse primers. The sequences of the target circRNA primers (Tsingke Biological Technology) are shown (**TABLE 1**).

TABLE 1. Sequences of target circRNA primers

CircRNA	Primer sequence-F	Primer sequence-R
circ*FGGY*	AGATTTGCTGGGATAAGGC	CCTGGTTGACTGGTA
	G	ATGGG
circ*DNM3*	CTGGCAAACTTCCCCAGACGGAGAGCAACTGTCC	
	T	CTGTAG
circ*MBOAT2*	CCTCACAGTGTGCCAAGTT	GCCCAAAAGGGTAGC
	A	AACTA
circ*CCDC9*	CCCCTGGAGGAGTCTGAG	TACTCGGCGATCTTCT
		CCAT
circ*LZIC*	GCAGGTGCTATACTCAGCC	TCCATCTCTGCTAACC
	A	TTGTCC
circ*RBM33*	AAGCTGAGTTGACAGAAG	TTCTTCTCCACGACC
	ACCA	GTTCC
circ*EIF3A*	TGACAAGGACCCTGAGAG	TCTCTGTCCCATTCAC
	AG	GTTCT
circ*CEP350*	GCACAGTTATCAAGTACAG	CACACTTTCCCATCA
	AATGC	GGTGTT
circ*SEC63*	TGGGAACGTGAATTCCAAACCATACCGGACTGGA	
	CT	TTCTC
GAPDH	CTTCATTGACCTCAACTAC	CTCGCTCCTGGAAGA
	ATGG	TGGTGAT

Reaction System Preparation

QX200 ddPCR Supermix (Bio-Rad) and DG Oil (Bio-Rad) were added to a DG8 cartridge (Bio-Rad), which was placed in a QX200 droplet generator for droplet generation. Then the droplets were transferred to a 96-well PCR plate, which was covered with aluminum foil sealing film and sealed with preheated PX1 heat sealer. After the ddPCR reaction, the number of droplets was calculated by QuantaSoft software.

circRNA-microRNA-mRNA Interaction Network Analysis

Using the TargetScan and miRTarBase databases, the microRNAs (miRNAs) that target circRNAs that might bind were predicted. Then, messenger RNAs (mRNAs) with tight binding sites were selected from the database for competing endogenous RNA network analysis, and the circRNA-miRNA-mRNA network diagram was drawn using bioinformatics software.

Target circRNA and Functional Enrichment Analysis of Downstream Target Genes

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway functional enrichment analysis of target circRNA and downstream target genes was conducted using DAVID technology.

Statistical Analysis

SPSS 22.0 and GraphPad Prism were used for data processing and statistical analysis. The *t*-test was used to analyze the differentially expressed circRNAs, and hierarchical cluster analysis was performed. To analyze the differences between the experimental group and the control group, the independent sample *t*-test was adopted. The differences in copy number of target circRNAs between preoperative and postoperative plasma were analyzed using the paired *t*-test. The χ^2 test was used to analyze the relationship between target circRNAs and clinicopathological indicators in patients with lung adenocarcinoma. GraphPad software was used to draw ROC curves. The area under the ROC curve (AUC) was calculated to evaluate the diagnostic efficacy of circRNA. All tests were 2-sided, and $P < .05$ was considered statistically significant.

Results

Differential Expression Analysis of Plasma circRNAs in Lung Adenocarcinoma Patients

We used high-throughput sequencing technology to analyze the plasma of 5 patients with early lung adenocarcinoma and 5 healthy controls. A total of 9310 circRNAs were detected. Differentially expressed circRNAs were identified based on the following criteria: fold change (FC) ≥ 2 and $P < .05$. We found that 9 circRNAs were upregulated and 62 circRNAs were downregulated. The 9 upregulated circRNAs were circ*FGGY*, circ*DNM3*, circ*MBOAT2*, circ*CCDC9*, circ*LZIC*, circ*RBM33*, circ*EIF3A*, circ*CEP350*, and circ*SEC63*. Then the circRNA sequence length (**FIGURE 1A**) and circRNA distribution on chromosomes (**FIGURE 1B**) were analyzed. The results showed that the length of circRNAs with differential expression ranged from 100 to 1200 nucleotides (nt). However, most circRNAs are between 100 and 500 nt in length. In addition, hierarchical cluster analysis was performed on the differentially expressed circRNAs. The thermal diagram, scatter diagram, and volcano diagram were drawn to show the results intuitively (**FIGURE 1C** and **1D**).

Screening and Analysis of Target circRNAs

To find potential markers for the diagnosis of lung adenocarcinoma, we conducted ddPCR analysis to determine the copy numbers of 9 upregulated circRNAs in the plasma of lung adenocarcinoma patients. We found that the copy numbers of circ*LZIC* (FC = 3.84, $P < .05$) and circ*CEP350* (FC = 2.55, $P < .05$) were increased in plasma in lung adenocarcinoma patients (**FIGURE 2A**). A literature review revealed that no roles for these 2 circRNAs in lung cancer have been reported, so we selected them as research objects.

The University of California Santa Cruz Genome Database (UCSC) and CircBase databases were used to obtain relevant information on these 2 circRNAs. circ*LZIC* (hsa_circ_0000014) is derived from the *LZIC* gene on chromosome 1 (Chr1: 9,991,948–9,994,918), and it is composed of 3 exons (exon 5, exon 6, and exon 7) of *LZIC*. circ*CEP350* (hsa_circ_0003942) is derived from the *CEP350* gene on chromosome 1 (Chr1: 179,972,308–179,975,702), and it consists of 2 exons (exon 7 and exon 8) of the *CEP350* gene. The UCSC database was used to analyze the mother genes of these circRNAs (**FIGURE 2B** and 2**C**).

The ddPCR Analysis of the Copy Numbers of Target circRNAs in Plasma

We performed ddPCR validation of plasma collected from 25 healthy people, 25 patients with early lung adenocarcinoma, and 25 patients with advanced lung adenocarcinoma. The results showed that the copy numbers of circ*LZIC* and circ*CEP350* were increased in the plasma of lung adenocarcinoma patients. The differences were highly statistically significant ($P < .001$), and the results were consistent with the sequencing results (**FIGURE 3A-3D**).

Then, we explored whether these circRNAs could be used as biomarkers to evaluate the efficacy of surgery. We analyzed the plasma of 3 patients with lung adenocarcinoma before and after surgery. We found that the copy numbers of circ*LZIC* and circ*CEP350* in plasma were significantly reduced after surgery (**FIGURE 3E** and **3F**).

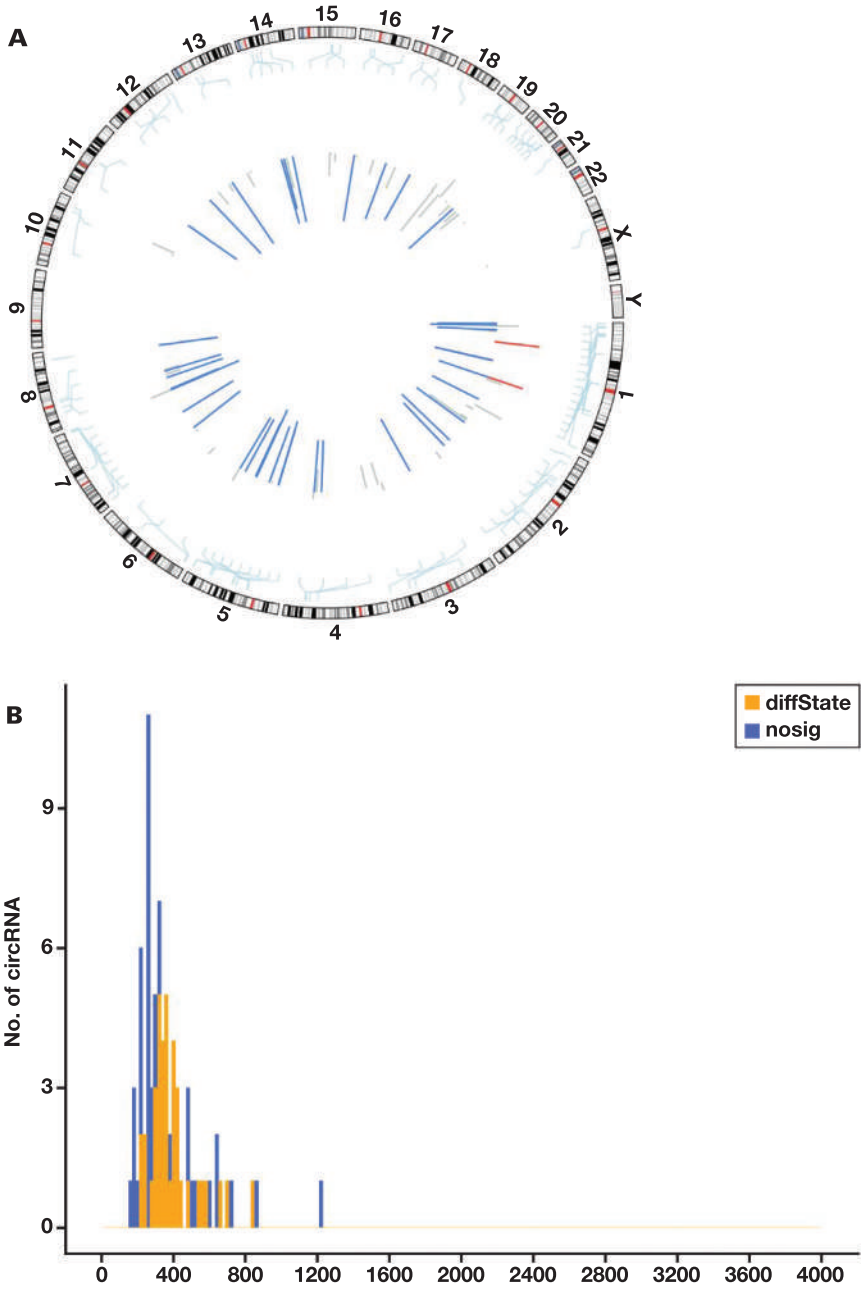
The Relationship Between Target circRNAs and Clinicopathological Parameters

According to the copy numbers of target circRNAs in plasma, lung adenocarcinoma patients were divided into a high expression group and a low expression group. Combined with clinicopathological data of patients with lung adenocarcinoma, we found significant correlation between circ*LZIC* copy number in plasma of patients with lung adenocarcinoma and TNM stage ($P = .045$) (**TABLE 2**). The plasma copy number of circ*CEP350* was significantly correlated with TNM stage ($P < .001$) and tumor size ($P = .023$) (**TABLE 2**). There was no significant correlation between copy number and gender, age, and lymph node metastasis (Supplemental Table 1). These results suggest that the plasma copy numbers of circ*LZIC* and circ*CEP350* may have diagnostic value.

ROC Curve Analysis of the Diagnostic Efficacy of Target circRNAs for Lung Adenocarcinoma

We constructed ROC curves to evaluate the efficacy of circ*LZIC* and circ*CEP350* as diagnostic markers of lung adenocarcinoma. The AUC value of circ*LZIC* was 0.782, and the sensitivity and specificity were 52% and 84%, respectively. The AUC value of circ*CEP350* was 0.764, and the sensitivity and specificity were 56% and 88%, respectively, showing high diagnostic value. Logistic analysis was conducted to test the efficacy of the combination of the 2 indicators as diagnostic

FIGURE 1. **Differential expression analysis of plasma circRNAs in lung adenocarcinoma patients. A, circRNA distribution on chromosomes. B, Length distribution of circRNAs. C, Heat map of differentially expressed circRNAs. D, Volcano map of differentially expressed circRNAs. Red points indicate upregulated circRNAs, green points indicate downregulated circRNAs.**



markers. When circ*LZIC* and circ*CEP350* were combined, the AUC value was 0.863, and the sensitivity and specificity reached 72% and 92%, respectively, showing this combination has extremely high diagnostic value and is of high clinical significance (**FIGURE 4A**). Then, ROC curve analysis was conducted to further evaluate the diagnostic efficacy of circ*LZIC* and circ*CEP350* as markers for early diagnosis of lung adenocarcinoma. The AUC value of circ*LZIC* was 0.786, and the sensitivity and specificity of circ*LZIC* were 72% and 64%, respectively. The AUC value of circ*CEP350* was 0.546, and the sensitivity and specificity were 64% and 56%, respectively. When circ*LZIC* and circ*CEP350* were analyzed together, the diagnostic efficacy was significantly improved; the AUC value was 0.803, and the sensitivity and specificity were 60% and 92%, respectively (**FIGURE 4B**).

Prediction of Downstream miRNAs and Target Genes of Target circRNAs

circRNAs bind to miRNAs through sponging, playing a role in the regulation of downstream target genes, which is the most frequently studied route. Through integrated analysis of CircBase, UCSC, and other databases, we found that circ*LZIC* and circ*CEP350* had binding sites with many miRNAs and screened out some miRNAs with strong binding ability to circ*LZIC* and circ*CEP350*. The 3 miRNAs with most binding

FIGURE 1. (cont)

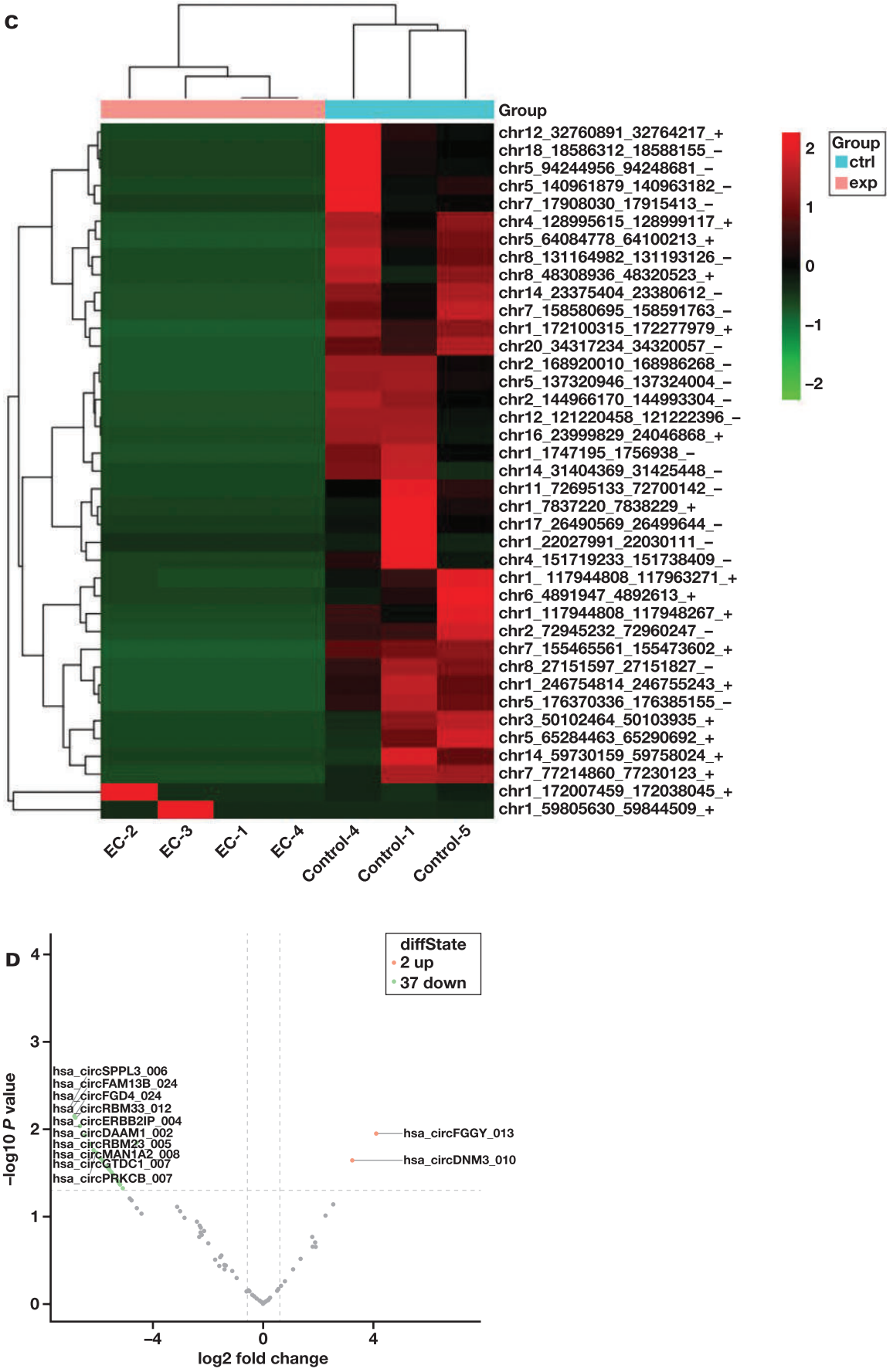
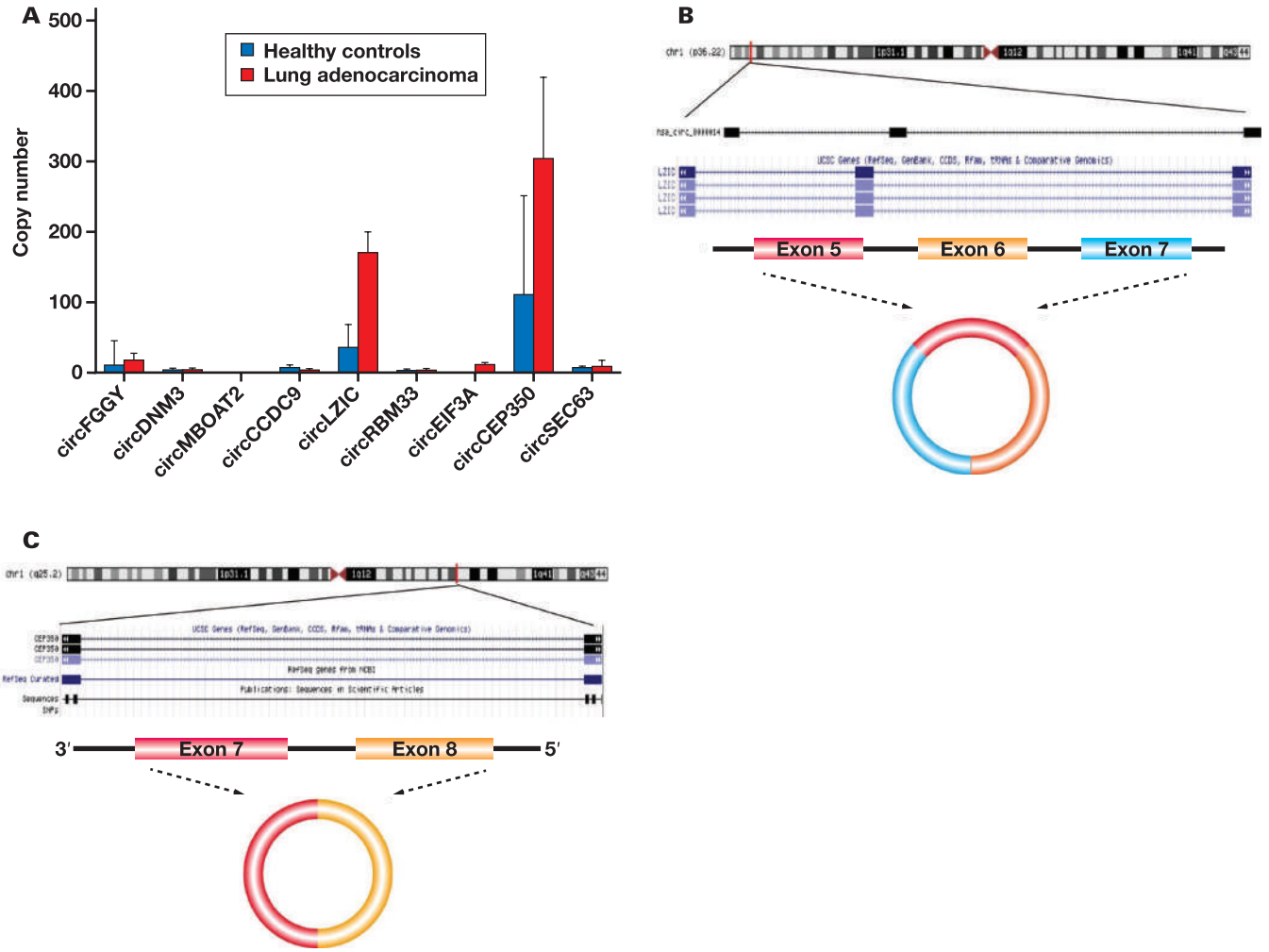


FIGURE 2. **Screening and analysis of target circRNAs. A, Difference in copy number of upregulated circRNAs in plasma of lung adenocarcinoma patients. B, circ*LZIC* source and maternal gene analysis. C, circ*CEP350* source and maternal gene analysis.**



sites with circ*LZIC* are miR-6504-5p, miR-1267, and miR-130b-5p. The 3 miRNAs with most binding sites with circ*CEP350* are miR-125a-5p, miR-125b-5p, and miR-4324. The interaction networks (**FIGURE 5A** and **5B**) were drawn using bioinformatics software.

GO and KEGG Pathway Analysis of Downstream Target Genes of circRNAs

We inserted circ*LZIC* and circ*CEP350* into miRbase, TangetMiner, and other databases, screened out downstream target genes with high correlation, and used the DAVID platform to perform GO functional enrichment analysis on downstream target genes. The enrichment of downstream target genes of circ*LZIC* exists in β-catenin binding, extracellular matrix components, and so on (**FIGURE 6A**). The enrichment of downstream target genes of circ*CEP350* exists in ubiquitin-like protein ligase binding, positive regulation of mitochondrial membrane permeability, positive regulation of mitochondrial membrane permeability involved in the apoptotic process, and so on (**FIGURE 6B**). Additionally, we conducted KEGG analysis of target circRNAs, downstream miRNAs, and target genes using the DAVID platform. We found that the main pathways in which circ*LZIC* was involved include the relaxin signaling pathway, dopaminergic synapses, and the neurotrophin signaling pathway (**FIGURE 6C**). The main pathways in which circ*CEP350* was involved included signaling pathways regulating pluripotency of stem cells, the Hippo signaling pathway, and pathways involved in colorectal cancer, pancreatic cancer, and axon guidance (**FIGURE 6D**).

Discussion

Liquid biopsy, as a noninvasive method to analyze circulating markers of tumors, can overcome the limitations of current tissue biopsies. Extracellular vesicles, circulating tumor DNA, and tumor-educated platelets are novel circulating markers of tumors.[21,22] Low abundance is a common drawback of liquid biopsies, but recent evidence suggests that circRNAs are much more abundant than mRNAs and miRNAs. Because of its high stability and disease specificity, circRNA circulates widely in various body fluids and exosomes and is easily detected. circRNA is considered as a potential ideal marker for liquid biopsy.[23]

Although many studies on the use of circRNAs in plasma and other body fluids as diagnostic markers have been conducted, few circRNAs

FIGURE 3. The copy numbers of target circRNAs in plasma were verified by droplet digital polymerase chain reaction (ddPCR). A, Copy numbers of circ*LZIC* in plasma. B, Copy numbers of circ*CEP350* in plasma. C, Copy numbers of circ*LZIC* in plasma as detected by RT-ddPCR. D, Copy numbers of circ*LZIC* in plasma as detected by ddPCR. E, circ*LZIC* copy numbers in plasma before and after surgery. F, circ*CEP350* copy numbers in plasma before and after surgery.

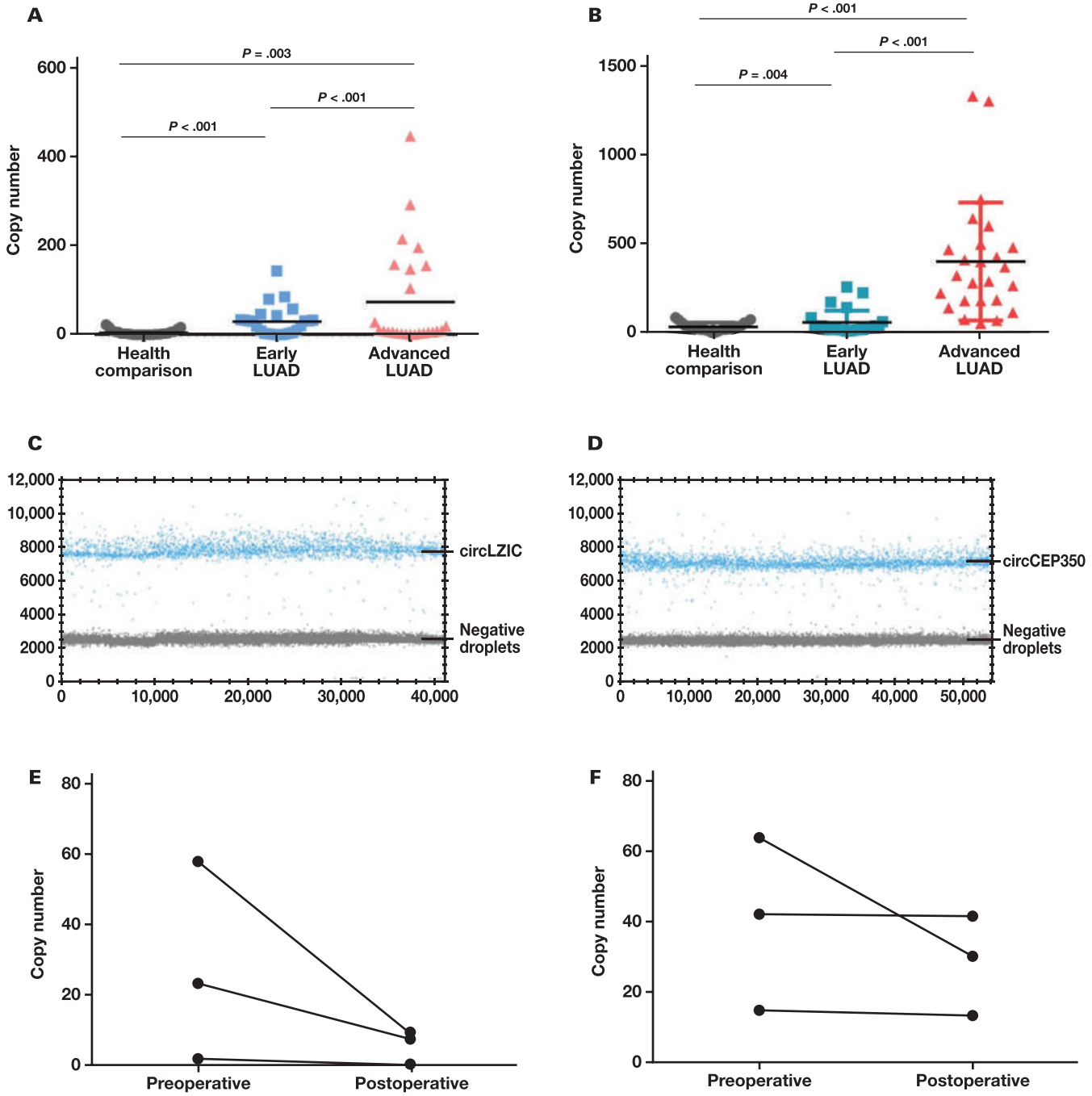


TABLE 2. Correlation of plasma circ*LZIC* and circ*CEP350* copy numbers with clinicopathological parameters

Clinicopathological parameter	circ*LZIC*			circ*CEP350*		
	High expression group	Low expression group	*P* value	High expression group	Low expression group	*P* value
Tumor size, cm						
≥3	22	11	.584	19	13	.023
<3	10	7		11	7	
TNM stage						
I-II	11	14	.045	11	14	<.001
III-IV	18	7		18	7	

FIGURE 4. Receiver operating characteristic (ROC) curves to evaluate the diagnostic efficacy of target circRNAs for lung adenocarcinoma. A, ROC curves of circ*LZIC* and circ*CEP350* for diagnosis of lung adenocarcinoma. B, ROC curves of circ*LZIC* and circ*CEP350* for early diagnosis of lung adenocarcinoma.

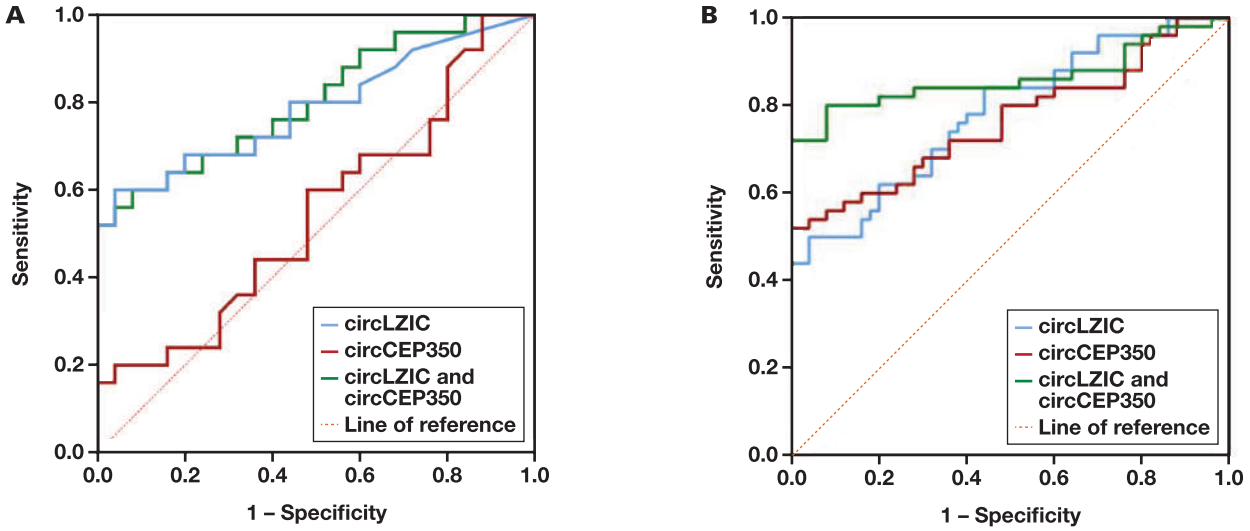
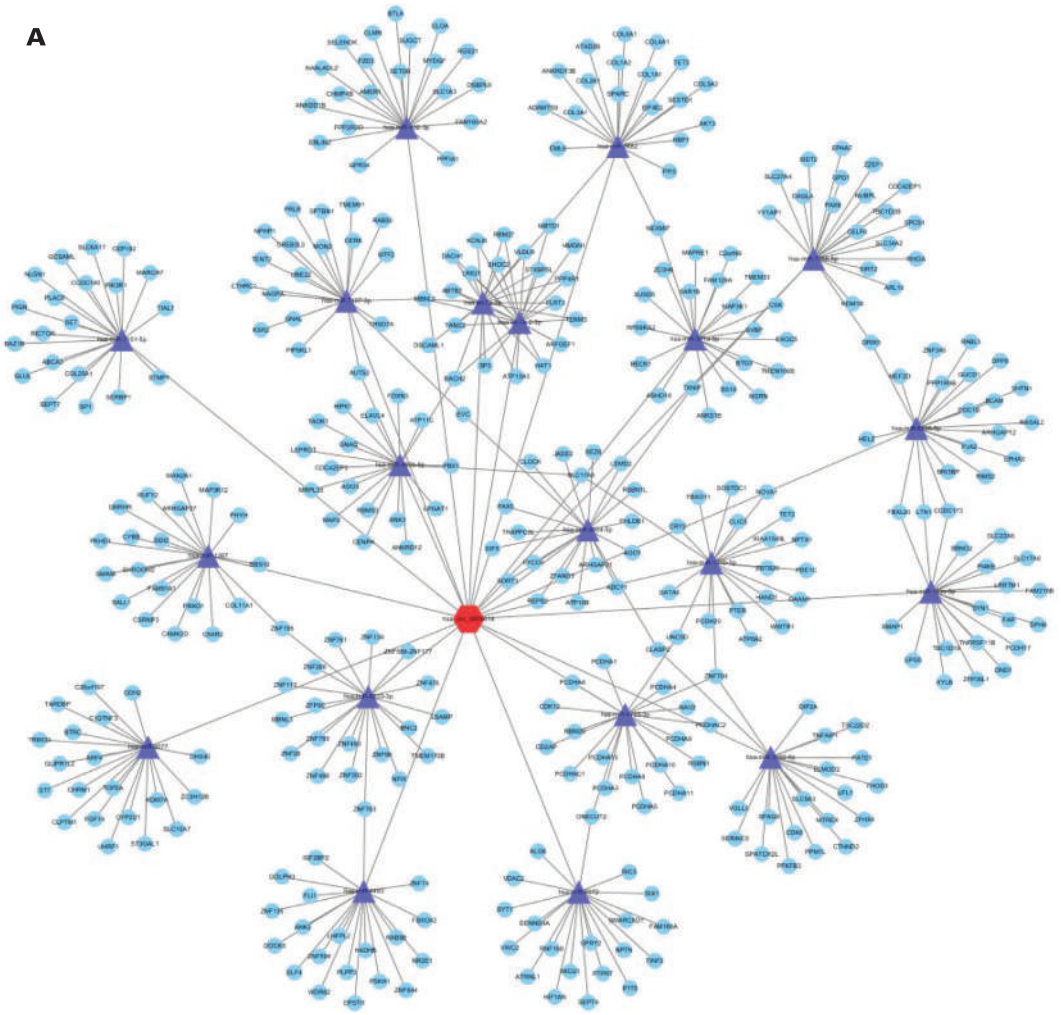


FIGURE 5. Prediction of downstream miRNAs and target genes of target circRNAs. A, Interaction network of circ*LZIC*, miRNAs, and downstream target genes. B, Interaction network of circ*CEP350*, miRNAs and downstream target genes.



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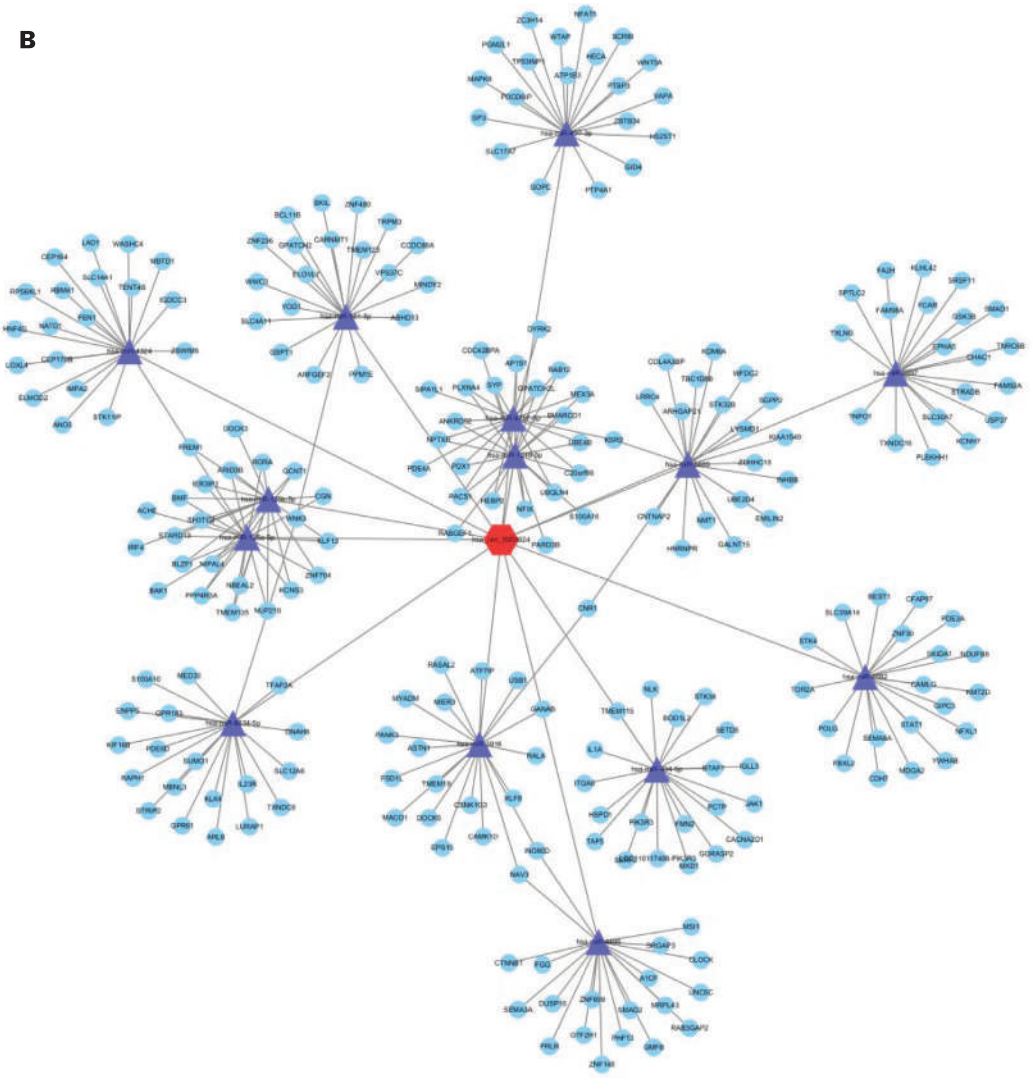


related to lung cancer have been identified, with low AUC values, and little attention has been paid to early lung cancer, thus failing to achieve an early screening effect.[24]

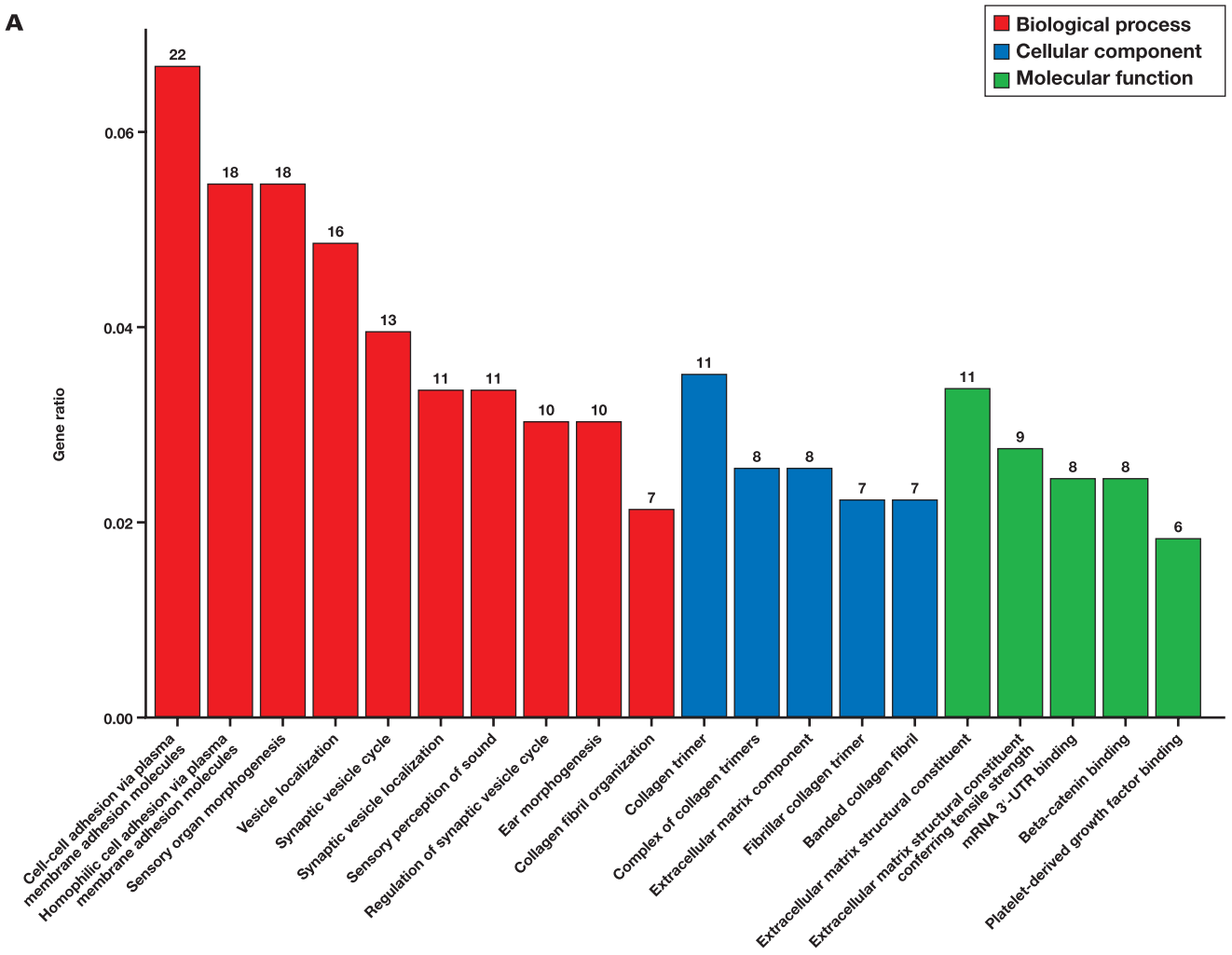
In many previous studies, qRT-PCR was used to detect circRNA because of its advantages of simplicity and intuition. However, qRT-PCR also has many limitations, among which the most significant one is the high false-positive rate when detecting nucleic acid. The detection effect of trace circRNA is not good,[25] which greatly affects the accuracy of qRT-PCR for plasma sample detection.[26] To reduce the experimental error caused by the above limitations, ddPCR was used in this study to quantitatively detect the copy number of circRNAs in plasma. Because ddPCR can achieve absolute quantitative detection and has high sensitivity, its stability for the detection of circRNA in plasma is more than 90% higher than that of qRT-PCR, and it is the best technique for the detection of trace circRNA in plasma samples.[27,28]

In this study, plasma analysis of lung adenocarcinoma patients and healthy controls by RNA sequencing revealed 9 upregulated circRNAs. We performed ddPCR to verify the upregulation of the 9 circRNAs, and it was found that the copy numbers of circ*LZIC* and circ*CEP350* in the plasma of lung adenocarcinoma patients were significantly higher than those of healthy controls.

Lung adenocarcinoma patients were divided into the high expression group and the low expression group according to the copy numbers of circ*LZIC* and circ*CEP350* in plasma. Further analysis of the relationship between these 2 circRNAs and clinicopathological parameters showed that there was significant correlation with TNM stage. The copy number of circ*CEP350* in plasma was significantly correlated with tumor diameter and TNM stage. These results suggest that circ*LZIC* and circ*CEP350* may be ideal biomarkers for (early) diagnosis of lung adenocarcinoma.

We used the CircBase and UCSC databases to analyze these 2 circRNAs. We found that the mother gene of circ*LZIC* is *LZIC*. Decreased *LZIC* expression could significantly promote the progression of renal cell cancer cells.[29] The mother gene of circ*CEP350* is *CEP350*. *CEP350* promotes DNA methylation in small cell lung cancer through centrosome mechanisms, relating to treatment resistance.[30] In conclusion, *circLZIC* and circ*CEP350* are closely related to the occurrence and development of malignant tumors and may serve as potential biomarkers of malignant tumors.

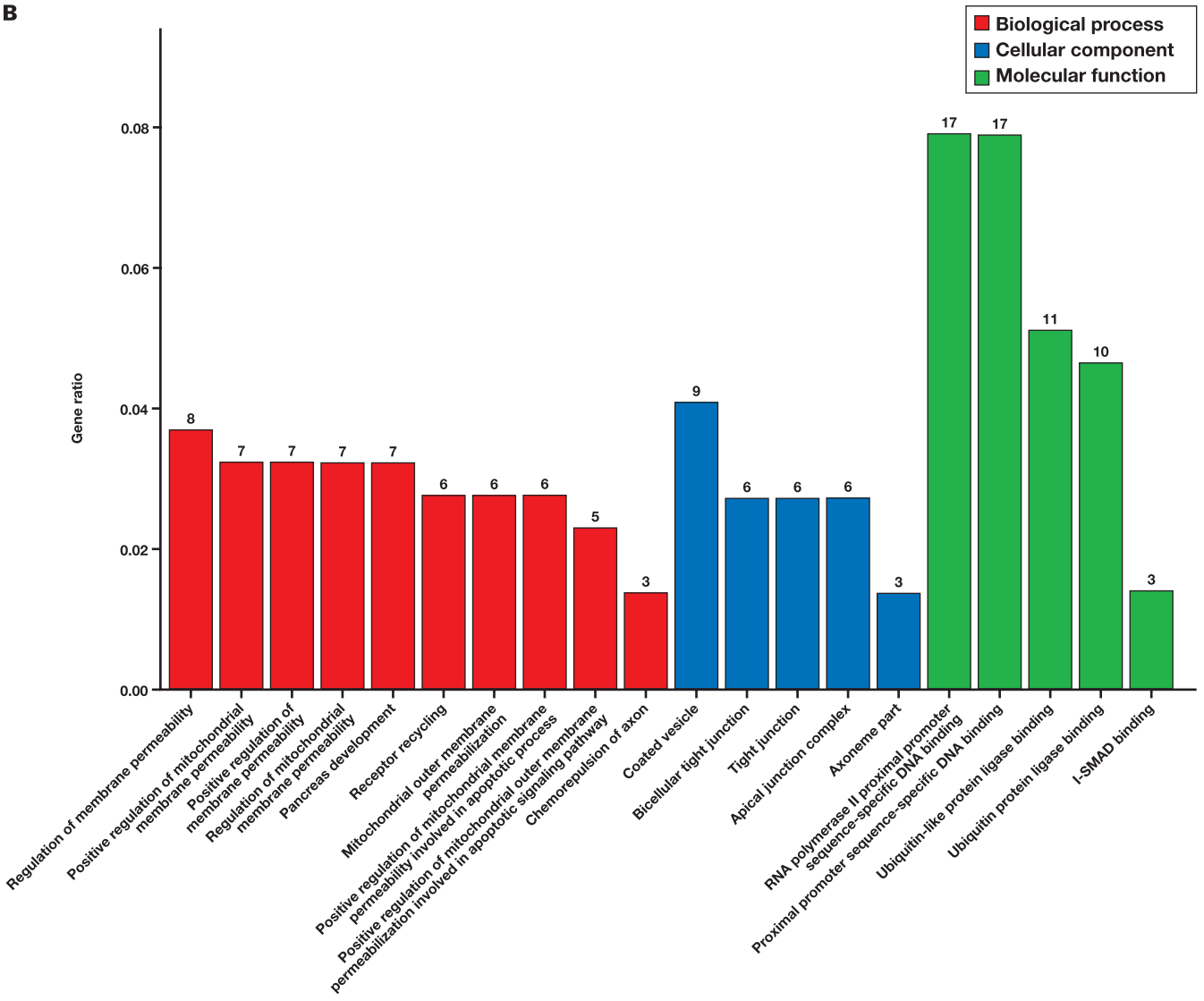
FIGURE 6. **Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of downstream target genes of target circRNAs. A, GO functional enrichment analysis of downstream target genes of circ*LZIC*. B, GO functional enrichment analysis of downstream target genes of circ*CEP350*. C, KEGG pathway enrichment analysis of downstream target genes of circ*LZIC*. D, KEGG pathway enrichment analysis of downstream target genes of circ*CEP350*.**



Recent studies have shown that some circRNAs are widely present in plasma, saliva, urine, and other body fluids and can be used as diagnostic and prognostic markers of diseases. He et al[31] found that upregulation of circRNA_0056616 was positively correlated with TNM stage and poor prognosis. Yan et al[32] found that hsa_circ_0001020 was significantly upregulated in the plasma of gastric cancer patients. To verify the diagnostic efficacy of circ*LZIC* and circ*CEP350* as diagnostic markers of lung adenocarcinoma, ROC curve analysis was performed for these 2 circRNAs. circ*LZIC* may indeed serve as a diagnostic marker of lung adenocarcinoma, with an AUC of 0.782. The AUC value of circ*CEP350* as a diagnostic marker for lung adenocarcinoma was 0.764. When the 2 indicators were combined, the AUC value reached 0.863, and the sensitivity and specificity reached 72% and 92%, respectively. When ROC curve analysis was conducted to examine the efficacy of circRNA as a diagnostic marker for early lung adenocarcinoma, the AUC value of circ*LZIC* was 0.786, and the AUC value of circ*CEP350* was 0.546. Analysis of circ*LZIC* and circ*CEP350* combined yielded an AUC value of 0.803 and a sensitivity and specificity of 60% and 92%, respectively. These results suggested that the combination of circ*LZIC* and circ*CEP350* may serve as an ideal biomarker for diagnosis of lung adenocarcinoma.

Extensive studies have shown that circRNAs exert their effects mainly by competitively binding with miRNA and reversely regulating the function of downstream mRNAs. To further explore the functional mechanisms of circ*LZIC* and circ*CEP350* in tumors, we analyzed miRNAs targeted to circ*LZIC* and circ*CEP350* through UCSC and other databases. The results showed that both circ*LZIC* and circ*CEP350* had many miRNA binding sites. We conducted GO analysis to examine the closely bound miRNAs and downstream target genes of circ*LZIC* and circ*CEP350*. The enrichment of downstream target genes of circ*LZIC* exists in β-catenin binding, extracellular matrix components, and so on. The enrichment of downstream target genes of circ*CEP350* exists in ubiquitin-like protein ligase binding, positive regulation of mitochondrial membrane permeability, positive regulation of mitochondrial membrane permeability involved in apoptotic processes, and so on. KEGG pathway analysis was performed on target circRNAs, downstream miRNAs, and target genes. The relaxin signaling pathway was found to be the most strongly enriched

FIGURE 6. (cont)



among downstream target genes of circ*LZIC*. The relaxin/*RXFP1* pathway plays an essential role in ovarian cancer tumorigenesis.[33] The Hippo signaling pathway was the most strongly enriched among circ*CEP350* downstream target genes. Previous studies have shown that the Hippo and Wnt pathways were linked by *KCTD11*, inhibiting the proliferation and migration of lung cancer cells.[34] Therefore, we preliminarily confirmed that circ*LZIC* and circ*CEP350* could target miRNA molecules to act on downstream pathways and played an important role in the occurrence and development of lung adenocarcinoma.

In conclusion, we used high-throughput technology to screen the changes in circRNA expression in plasma of patients with early lung adenocarcinoma and found some highly expressed circRNAs. circ*LZIC* and circ*CEP350* were found to be potential biomarkers for diagnosis, especially early diagnosis, of lung adenocarcinoma. In addition, we predicted that circ*LZIC* and circ*CEP350* have the potential to regulate the occurrence and development of lung cancer by binding miRNAs through bioinformatics analysis. This study provided a new way to find biomarkers for diagnosis of lung adenocarcinoma.

Conclusion

The plasma copy numbers of circ*LZIC* and circ*CEP350* were increased in patients with lung adenocarcinoma and significantly correlated with TNM stage. circ*LZIC* downstream target genes were enriched in the relaxin signaling pathway. circ*CEP350* downstream target genes were enriched in the Hippo signaling pathway, which was closely related to tumor development and may serve as an ideal molecular marker for diagnosis, especially early diagnosis, of lung adenocarcinoma.

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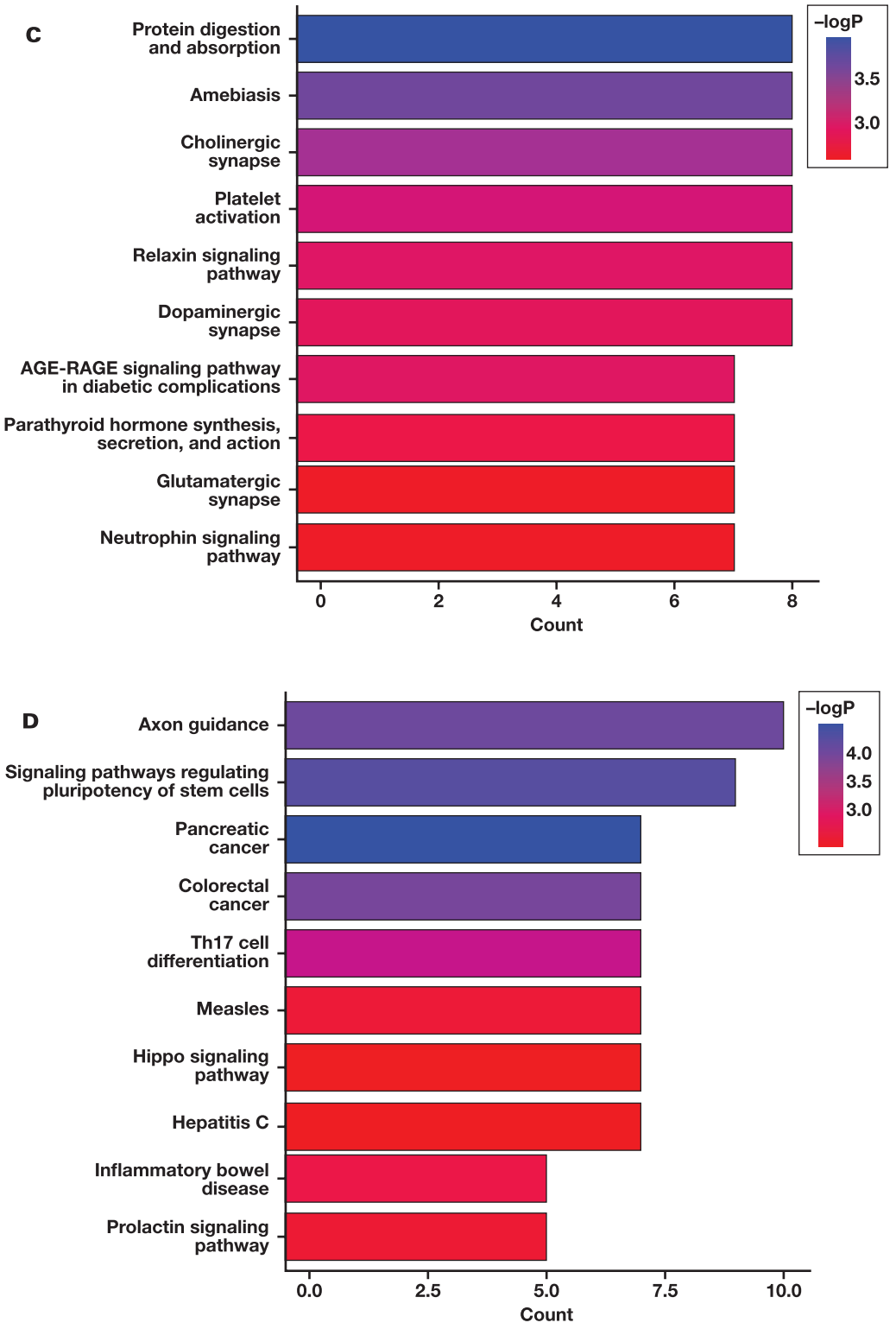
FIGURE 6. (cont)



Conflict of Interest Disclosure

The authors have nothing to disclose.

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Frequency of serological markers of rheumatoid arthritis in patients with Hashimoto's thyroiditis

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Key Words: Hashimoto's thyroiditis, rheumatoid arthritis, anti-cyclic citrullinated peptides antibodies, rheumatoid factor, adults, Tunisia

Abbreviations: HT, Hashimoto's thyroiditis; AID, autoimmune disease; TPO-Ab, autoantibodies against thyroid peroxidase; TG-Ab, autoantibodies against thyroglobulin; AITDs, autoimmune thyroid diseases; RA, rheumatoid arthritis; RF, rheumatoid factor; CCP-Ab, anti–cyclic citrullinated peptides antibodies; ATA, antithyroid antibodies; RR, relative risk

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ABSTRACT

Background: Hashimoto's thyroiditis (HT) is an autoimmune disease that is frequently associated with other autoimmune conditions.

Objective: To perform serological screening for rheumatoid arthritis (RA) in patients with HT.

Methods: Our study included 88 consecutive serum specimens of patients with confirmed HT and 88 sex- and age-matched healthy subjects. All study participants were tested for anti-cyclic citrullinated peptides antibodies (CCP-Ab) and rheumatoid factor (RF). CCP-Ab and RF were performed using ELISA commercial kits. Statistical analysis was conducted using Epi Info, version 3.

Results: Out of 88 patients with HT, 15 (17.0%) had CCP-Ab or RF. The frequency of serological markers of RA was significantly higher in patients than in control individuals (17.0% vs 4.5%; $P = .007$). RF was more frequent in patients than in the control group, and the difference was statistically significant (13.6% vs 3.4%; $P = .01$). Isolated RF-IgM was absent in all controls and present in 6 patients with HT (6.8% vs 0%; $P = .02$). Out of 14 male patients, 3 (21.4%) had antibodies of RA. There was no significant difference in age between patients with CCP-Ab or RF and those without.

Conclusion: A high frequency of serological markers of RA was highlighted in patients with HT.

Introduction

Hashimoto's thyroiditis (HT) is an organ-specific autoimmune disease (AID). It is characterized by the presence of autoantibodies against thyroid peroxidase (TPO-Ab) and thyroglobulin (TG-Ab). The clinical presentation of HT is hypothyroidism secondary to thyroid gland destruction, due to a high inflammatory load and apoptosis occurring because of a cellular immune response and loss of immune tolerance.[1]

Autoimmune thyroid diseases (AITD), particularly HT, are known to be risk factors for osteoarthritis.[2-4] The findings of a study of patients with TPO-Ab and/or TG-Ab and normal thyroid function demonstrated that 98% of patients had arthralgia and 88% had arthritis.[5] Undifferentiated inflammatory arthropathy was reported in 25% of patients with HT.[6] Nonspecific rheumatic manifestations were demonstrated in 20% of patients with HT.[7] Arthritis could reveal HT and could disappear under replacement therapy.[8]

The immunopathogenesis of articular manifestations observed in HT is not well known. However, an association of rheumatoid arthritis (RA) to HT could explain this clinical picture.[9-12]

RA is a systemic AID characterized by chronic synovial inflammation and joint damage. In severe cases, a loss of joint function and disability could occur. The clinical picture of RA includes morning stiffness, joint swelling, and pain. The immunological markers of RA are rheumatoid factor (RF) and anti–cyclic citrullinated peptides antibodies (CCP-Ab).[13]

The relationship between RA and HT could be explained by many factors:

1. Genetic susceptibility could play a role in the connection between RA and HT. A study of 202 Tunisian patients with HT revealed an association between $IL1RN^{VNTR}$ polymorphism and the production of TPO-Ab at the onset of HT.[14] We note that IL-1 is one of the key

elements in the immunopathogenesis of RA. IL-1 causes not only inflammation of the joints but also systemic inflammation.[13]

2. Smoking is known to trigger HT[15] and RA.[16] In our country, Tunisia, smoking occurs at among the highest rates worldwide.[17]
3. Hepatitis C virus infection was reported to have a high degree of association with RA.[18] Hepatitis C virus is also a risk factor for HT.[1] In Tunisia, 3.32% of the population has chronic hepatitis C.[19]
4. Vitamin D deficiency, which is a risk factor for HT[1,20] and RA,[21] occurs very frequently in Tunisia (47.6%).[22]
5. Iron-deficiency anemia had been demonstrated to be a risk factor for HT[1] and RA[23] and was reported to be very common in Tunisia (30%).[24]
6. HT and RA occur frequently. HT is among the most common organ-specific types of AID: its incidence is between 0.3 and 1.5, and women are 9 times more often affected than men.[25] RA is the most common inflammatory arthropathic disease worldwide. Its incidence is almost 0.5 and is also related to sex: women are affected 2 to 3 times more often than men.[9]

All of these factors—ie, a high frequency of articular manifestations in HT and plenty of risk factors for HT in Tunisia—prompted us to do a serological screening of RA in HT, especially because only 2 studies determined the frequency of serological markers of RA in HT,[26,27] and none of them performed the determination of 3 isotypes of RF. We also aimed to explain the association between RA and HT.

Methods

Study Populations

Specimen Collection

Serum specimens from patients with HT were collected during a 14-month period from 4 hospitals in the center of Tunisia. Specimens consisting of 4 mL of venous blood were received in our laboratory for TPO-Ab and TG-Ab assay. Serum from these specimens was collected by centrifugation at 3000 rpm for 15 minutes, and enzyme-linked immunosorbent assay (ELISA) was carried out for antithyroid antibodies (ATA) testing. A total of 400 µL of serum testing positive for TPO-Ab and/or TG-Ab was aliquoted and stored at −80°C until use for analysis.

Specimen Selection

The following individuals were excluded from our study:

- Children
- Patients with associated autoimmune diseases
- Patients being treated for hypothyroidism

Control Group

A total of 88 sex- and age-matched healthy subjects with negative ATA results served as a control group.

All patients and control individuals were tested for serological markers of RA. The ethical committee of our hospital gave its approval for this study.

Antithyroid Antibodies

Antithyroperoxydase Antibodies

Detection of TPO-Ab was performed using a commercial ELISA kit (Euroimmun; PerkinElmer Germany Diagnostics) providing a quantitative assessment of human IgG TPO-Ab. TPO was bound to microwells and served as an antigen. A total of 100 µL of each diluted specimen (1/201 in the sample buffer provided with the kit) was incubated in the wells. For specimens testing positive, TPO-Ab bound to the immobilized TPO antigen. After incubation, a washing step was carried out. A second incubation with peroxidase-labeled anti–human IgG antibodies was performed. After incubation, a second washing step eliminated the unbound enzyme conjugate. A third incubation, with substrate solution containing a chromogenic substrate, was performed. Finally, the addition of an acid (H_2SO_4) stopped the reaction and generated a yellow end product. The intensity of the resulting color was proportional to the concentration of TPO-Ab in the serum specimens. The optical density of this color reaction was measured at a wavelength of 450 nm. The cutoff value for positivity was 50 IU/mL.

Antithyroglobulin Antibodies

TG-Ab detection was performed using a commercial ELISA kit (Euroimmun). The kit provided a quantitative assay for human TG-Ab of the IgG isotype. Diluted specimens (1/201 in the sample buffer provided with the kit) were incubated in the wells coated with TG antigen. If specimens contained TG-Ab, specific IgG antibodies were bound to the immobilized TG antigen. After incubation, a washing step was carried out. A second incubation with peroxidase-labeled anti–human IgG was performed. A washing step removed unbound enzyme conjugate. The bound enzyme conjugate catalyzed a color reaction when the substrate solution, containing a chromogenic substrate, was added. The addition of an acid stopped the reaction, generating a yellow end product with intensity proportional to the concentration of TG-Ab in the serum. The optical density of the color intensity was measured at a wavelength of 450 nm. The cutoff for positivity was 100 IU/mL.

Serological Markers of Rheumatoid Arthritis

Anti-Cyclic Citrullinated Peptides Antibodies

CCP-Ab were detected using a second generation ELISA test (Orgentec Diagnostika; Sebia). The assay kit provided a quantitative assessment of human CCP-Ab of the IgG isotype. The kit uses highly purified cyclic citrullinated vimentin peptides bound to microwells and served as an antigen. For each specimen, the serum was diluted at 1/101 in the sample diluent provided with the kit, and 100 µL was incubated in the wells. If the specimen contained CCP-Ab of the IgG isotype, these specific antibodies were bound to the immobilized antigen. After 30 minutes of incubation, a washing step was performed to remove the unbound antibodies, and then a second 30 minutes of incubation of peroxidase-labeled anti–human IgG antibodies was carried out to detect the antibody-antigen complexes formed.

After incubation, a second washing step was performed to eliminate the unbound enzyme conjugate. A color reaction was initiated by adding a substrate solution, containing a chromogenic substrate, catalyzed by the peroxidase enzyme. Finally, the addition of the stop solution stopped the reaction and generated a yellow end product. The intensity of the resulting yellow color is proportional to the concentration of CCP-Ab in the serum specimens. The optical density of this color reaction was measured at a wavelength of 450 nm. The cutoff value for positivity was set at 20 U/mL.

Rheumatoid Factor

RF-Ab were assessed with a commercial ELISA kit (Orgentec Diagnostika). The kit provided a quantitative assay for human RF-Ab

of the IgM, IgG, and IgA isotypes. The wells of the microplates were coated with Fc fragment of human IgG1. Patient specimens were diluted at 1/101 in the sample diluent provided with the kit, and 100 µL was incubated in the wells for 30 minutes. If specimens contained RF-Ab, specific IgM, IgG, or IgA antibodies were bound to the immobilized antigen. After incubation, a washing step was performed, and subsequently added peroxidase-labeled anti–human IgM, IgG, or IgA was bound to the antibody-antigen complexes formed with each isotype. A second washing step removed unbound enzyme conjugate. The bound enzyme conjugate catalyzed a color reaction when the substrate solution, containing a chromogenic substrate, was added. The addition of an acid stopped the reaction, generating a yellow end product. The intensity of the resulting color is proportional to the concentration of RF-Ab of each isotype in the serum. Finally, the optical density of the color intensity was measured photometrically, at a wavelength of 450 nm. The cutoff values for positivity were 49.5 U/mL, 45.5 U/mL, and 31 IU/mL for RF-IgG, RF-IgA, and FR-IgM, respectively.

Statistical Analysis

Statistical analysis was conducted using Epi Info software, version 3. To compare frequencies of serological markers of RA in patients and in controls, we used χ^2 testing as part of Fisher exact testing. A P value of <.05 was considered significant. For significant results, confidence interval and odds ratio were determined using WinPepi software.

Results

Our study cohort included 88 untreated patients with HT (age: mean [SD], 44.4 [15.7] years; sex ratio: F/M: 74/14) and 88 controls (43.2 [11.6] years; sex ratio F/M: 74/14). Of the 88 patients with HT, 15 (17.0%) had CCP-Ab or RF. The frequency of serological markers of RA was significantly higher in patients than in controls (17.0% vs 4.5%; P = .007). RF was more frequent in patients than in the control group, and the difference was statistically significant (13.6% vs 3.4%; P = .01). Isolated RF-IgM was absent in all controls and present in 6 patients with HT (0% vs 6.8%; P = .02) (**TABLE 1**). There was no significant difference in frequencies of RF and CCP-Ab between men and women. CCP-Ab, RF, and RF-IgA were more frequent in males than in females (14.3% vs 5.4%, P = .4; 21.4% vs 12.2%, P = .5; and 14.3% vs 4.1%, P = .3, respectively) but the difference did not rich significance levels. In male patients, RF-IgA was more frequent than RF-IgG (14.3% vs 0.0%, P = .4). Of 14 male patients, 3 (21.4%) had antibodies of RA. All of them had RF, and 2 had CCP-Ab and RF. Levels of RA serological markers for the 15 patients with positive results and the epidemiological characteristics of these patients are shown in **TABLE 2**. We compared the mean ages between the 2 groups of patients with serological markers of RA and those without these autoantibodies, and we did not find a statistically significant difference.

Discussion

Herein, we demonstrated a higher frequency of serological markers of RA in patients with HT compared with controls (17.0% vs 4.5%; P = .007). In total, 3 patients out of 88 had CCP-Ab and RF; this frequency was similar to that reported by Elnady et al[26] (3.4% and 3.3%, respectively). The frequency of CCP-Ab and the frequency of RF were higher in the study results of Elnady et al[26] than in ours (19.7% vs 6.8% and 34.4% vs 13.6%, respectively).

The differences between our study findings and those of Elnady et al[26] could be explained by the method used for the detection of RA autoantibodies. We used ELISA for CCP-Ab and RF, whereas Elnady et al[26] used chemiluminescence-microparticles immunoassay. The study populations were also different. Our cohort included only adults (mean age, 44.4 years), but Elnady et al[26] included children, adolescents, and adults (mean age, 26.98 years). Also, we investigated separately 3 isotypes of RF, whereas Elnady et al[26] determined the global frequency of RF without determining the frequency of each isotype. In another study,[27] only RF-IgM has been determined in patients with HT, and the frequency was similar to that in our study findings (5.7% and 7.9%, respectively).

RF and CCP-Ab have a high positive predictive value for RA.[28] Besides, Elnady et al[26] conducted a 2-year follow-up of patients with HT who had serological markers of RA, and they found that 45% of those patients developed RA. Among the antibodies, CCP-Ab is the more specific, compared with RF-IgG, IgM, and IgA.[29] In the present study, CCP-Ab were present in 6 of 88 patients with HT (6.8%). These patients could develop RA in the future.

The frequency of RA has been determined in other studies by data collection from medical records. In fact, Boelaert et al,[11] who included 495 patients with HT in their cohort, demonstrated that 4.24% of them had RA, and that the relative risk (RR) for RA was 4.38 in females

TABLE 1. Serological markers of rheumatoid arthritis in patients with Hashimoto's thyroiditis

Analyte	Patients (n = 88), No. (%)	Control group (n = 88), No. (%)	OR (95% CI)	*P* value
CCP-Ab or RF	15 (17)	4 (4.5)	4.32 (1.29-18.52)	.007
CCP-Ab and RF	3 (3.4)	0		.20
CCP-Ab	6 (6.8)	1 (1.1)		.10
CCP-Ab only	3 (3.4)	1 (1.1)		.60
RF	12 (13.6)	3 (3.4)	4.47 (1.14-25.43)	.01
RF-IgG	0	1 (1.1)		NS
RF-IgA	5 (5.7)	0		.05
RF-IgM	7 (7.9)	1 (1.1)		.06
RF-IgA only	3 (3.4)	0		.20
RF-IgM only	6 (6.8)	0		.02

CCP-Ab, anti–cyclic citrullinated peptides antibodies; OR, odds ratio; RF, rheumatoid factor.

TABLE 2. Epidemiological characteristics and levels of serological markers of rheumatoid arthritis antibodies in patients testing positive for the disease[a]

No.	Sex	Age, y	TPO-Ab (NV<50 IU/mL)	TG-Ab (NV <100 IU/mL)	CCP-Ab (NV<20 U/mL)	RF-IgG (NV<49.5 U/mL)	RF-IgA (NV<45.5 U/mL)	RF-IgM (NV<31 IU/mL)
1	M	55	469	132	**55.7**	15.4	11.4	**33.2**
2	F	39	—	542	6.6	34.1	13.1	**63.6**
3	M	62	—	138	4.7	8	**51.5**	0
4	F	35	>500	—	**51.4**	4.9	5.5	2.5
5	F	36	>500	206	**26.3**	7.9	**55.9**	0
6	F	55	>500	544	5	9.5	13.6	**56.2**
7	F	64	—	189	**177.3**	7.5	7.0	0.9
8	F	34	—	244	3.5	14.9	12.2	**68.9**
9	F	47	>500	—	6.2	14	13.9	**114.5**
10	F	21	190	—	**21.8**	5.6	5.5	0
11	F	38	>500	685	12.2	20.1	8.4	**69.2**
12	F	53	>500	—	3.9	11.6	**174.6**	2.8
13	F	26	>500	294	10.1	47	37.9	**169.1**
14	M	59	>500	—	**52.6**	15.4	**46.8**	6.6
15	F	60	—	364	7.5	11.2	**46.6**	22.9

CCP-Ab, anti–cyclic citrullinated peptide antibodies; IU, international unit; NV, normal value; RF, rheumatoid factor; TG-Ab, autoantibodies against thyroglobulin; TPO-Ab, autoantibodies against thyroid peroxidase.
[a]*Bold indicates values outside normal range.*

and 3.13 in males. Also, these researchers found that RA was the most common AID associated with HT. In a meta-analysis study that included 9512 patients with HT, it has been reported that 2.38% of the patients with HT had RA.[12] We were intrigued to discover, in another meta-analysis, that the opposite finding was realized, which indicated that patients with RA had a high risk of developing thyroid dysfunction, especially hypothyroidism.[30]

Mahagna et al,[15] who performed a database investigation, reported findings opposite to ours in a very large series of patients with RA (n = 11,782). These investigators found that 16% of patients with RA had hypothyroidism; this frequency was similar to that of CCP-Ab or RF (17.0%) in our patients with HT. We mention also that Li et al[31] demonstrated, in a case-control study, that 26.2% of patients with RA had hypothyroidism; they also reported, as determined by meta-analysis, a strong correlation between overt hypothyroidism and RA (pooled RR, 5.31). So, RA was considered to be a high risk factor for thyroid dysfunction. Anoop et al[32] conducted a cross-sectional prospective study of 100 patients with RA and found that 22% had thyroid dysfunction and 31% had TPO-Ab. Among the 22 patients with thyroid dysfunction, 15 had hypothyroidism: 5 had it detected before diagnosis of RA, 5 detected after diagnosis of RA, and 5 detected concurrently with RA.

AITD increases the risk of RA and vice versa. AITD could precede, coincide with, or follow RA. Moreover, AITD could affect the disease activity or the response to RA treatment.[33-35] So, there is cross-talk between the thyroid gland and the joints. In fact, Chen et al[34] demonstrated a correlation between ATA and radiographic joint damage in patients with RA and suggested an important role of ATA in joint damage. They also hypothesized that ATA are produced locally in the joints because of the infiltration of a high number of plasma cells in the synovial tissue. Moreover, Blake et al[36] detected TPO-Ab and TG-Ab in the synovial fluid of 21 of 37 patients (56.7%) with seropositive RA. They also demonstrated the absorption of TG-Ab activity by human thyroglobulin. A thyroid hormone network in synovial fibroblasts has been described.[37] In addition, thyroid hormone actions have been reported in cartilage and bone, and thyroid receptor are expressed in chondrocytes.[38] Also, TSH was demonstrated to downregulate autophagy and to promote apoptosis in chondrocytes.[39]

The association between HT and RA could be explained by 2 common features:

1. Smoking is an environmental trigger for both HT[15] and RA.[16] In Tunisia, smoking is among the highest worldwide and it is more frequent in men than in women. The frequency of smoking among men in Tunisia is 50.4%.[17] In the present study, the frequency of CCP-Ab was more frequent in men than in women with HT (14.3% vs 5.4%). RF was also more frequent in men than in women (21.4% vs 12.2%), but the difference did not reach statistically significance levels because of the low number of male patients with HT (14/88 [15.9%]). In fact, HT, like RA, has a female preponderance, explained by skewed X-chromosome inactivation in females.[40] Moreover, RF-IgA, which was described to be associated with smoking in patients with African ethnic ancestry, was more frequent in males than in females (14.3% vs 4.1%). Besides, in male patients with HT, RF-IgA was more frequent than RF-IgG. We have previously undertaken a serological screening for RA in patients with celiac disease and have also found that RF-IgA was much higher than RF-IgG, and the difference was statistically significant.[41] We have also previously demonstrated that RF-IgA was more specific for RA than RF-IgG.[29]
2. Increased intestinal permeability was demonstrated not only in HT[42,43] but also in RA.[44,45] Besides, increased zonulin serum concentration and positive correlation between zonulin concentration and IFN-γ systemic levels were reported in HT.[42] Also, in RA, high zonulin levels were reported.[45] The increased serum zonulin results in a leaky intestinal barrier, gut microbiota dysbiosis, and intestinal inflammation. Indeed, a high level has been reported of

IL-17 in HT[46] and of IL-1 and TNF-α in RA.[13] Intestinal barrier dysfunction was reported to play an integral role in the pathology of arthritis because bacteria or inflammatory cells will move from the gut to the circulation.[47]

In conclusion, RA occurs frequently in patients with HT. Endocrinologists should be aware of the etiology of arthralgia and arthritis in patients with HT. An early diagnosis of RA could help patients avoid joint damage and disability.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Vortexing specimens to disaggregate platelet clumps in EDTA specimens

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Key words: platelet clumps; vortexing; patient safety; cost containment; processing of EDTA specimens; reducing specimen recollections

Abbreviation: CAP, College of American Pathologists

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ABSTRACT

Objective: To compare platelet count results of specimens that yield platelet clump flags to platelet count results on these specimens after vortexing.

Method: Specimens that generated platelet count flags on Sysmex XN 3000 instruments were vortexed and rerun. Only data from specimens demonstrating elimination of platelet clump flags were used in this study. Pearson *r* analysis was performed on data.

Results: Comparison of complete blood count results (white blood cell count, red blood cell count, hemoglobin, hematocrit, and platelet count) all yielded Pearson *r* scores >0.9.

Conclusion: Additional patient comfort and safety concerns, as well as concerns over additional specimen collection and processing costs, may be avoided by vortexing and rerunning specimens flagged for platelet clumps when the platelet count is normal.

Introduction

Patient safety and the increasing costs of health care prompted this investigation. One of the major concerns in ensuring patient safety is re-collection of specimens. Re-collecting specimens can add to patient discomfort and decreased satisfaction with health care providers, delay in treatment due to prolonged turnaround times, and misdiagnosis or lack of treatment due to test abandonment.[1]

Re-collecting specimens also contributes to rising costs in delivering health care. Having specimens recollected, processed, and retested incurs additional costs to the hospital or facility where testing is performed.[1] Re-collection and processing costs are not reimbursed by insurance providers or patients.[2]

One reason for re-collection of patient specimens is spurious platelet counts, whether falsely elevated or falsely decreased, each due to a variety of causes.[3] Re-collection of specimens also occurs due to platelet clump flags, even when the platelet counts are in the normal reference range. However, these flags may not be reliable because some instruments often generate inaccurate platelet clumping flags as often as 20% of the time.[4,5] Having a specimen re-collected to verify platelet count for specimens that are flagged for platelet clumping may not always be necessary, especially for platelet count results in the normal range.

Gulati et al[6] found that vortexing specimens may disaggregate platelet clumps and thus remove platelet clump flags, allowing the results to be reported. Vortexing of specimens for 1 to 2 minutes, with a vortex setting of 8 to 10, was adequate to disaggregate platelet clumps. If the clumps are large enough to be counted as WBCs, this process results in increasing the platelet counts and decreasing WBC counts; the RBC counts are not affected. Specimen re-collection is only needed if "vortexing is unsuccessful or not feasible."[6] The purpose of this study was to validate the use of vortexing specimens with normal platelet counts that were flagged for platelet clumps, in an effort to resolve the platelet clump flag and provide for quicker turnaround times, along with avoiding specimen re-collection.

Methods

Four Sysmex XN 3000 instruments (Sysmex Corporation) were used to perform analysis of all specimens used in this study. Three levels of control material are run daily, to monitor the reproducibility of results from each instrument. Also, interinstrument comparisons are performed triannually, using College of American Pathologists (CAP) quality cross-check survey FH9Q. EDTA specimens for which a complete blood count (CBC) was ordered and which yielded platelet clump flagging were allowed to be loaded into the Sysmex SP-10 instrument for smear preparation and staining. Specimens were then vortexed using an SP Vortex Mixer (CAT-S8223) for 30 seconds to 1 minute at a setting of 9, allowed to rest for 2 minutes to settle any bubbles formed, and rerun. The prevortex and postvortex reports were printed, and the data were used in a correlation analysis (Pearson *r*) of results for red blood cell (RBC), white blood cell (WBC), hemoglobin (Hb), hematocrit (Hct), and platelet counts. The normal platelet count reference range used for this study is 145 to 445 × 10^3/μL.

Exclusions and Limitations

Only specimens that demonstrated disappearance of the platelet clump flagging with vortexing were used in this study (n = 97). Although only 71 of the specimens used in this study produced results in the normal range, all 97 specimens were included, to provide for better statistical validity. An additional 17 specimens that had yielded platelet clump flagging on the pre- and postvortex specimens were not included in this study, regardless of whether platelet counts were in the normal range; however, these were submitted for re-collection per institutional protocol. No comparisons were made with re-collected specimens because these often occurred during the following work shift. Comparison of re-collected specimen results to the results obtained on vortexed specimens that had initially been flagged for platelet clumps could be included in future studies. Another limitation is that not all specimen smears were reviewed after vortexing. Examination of all smears before and after vortexing is suggested for future studies.

Results

A total of 97 specimens that initially yielded platelet clump flagging were used for this study. In all, 11 specimens had decreased platelet counts before and after vortexing. Also, 11 specimens had decreased prevortex platelet counts and normal postvortex platelet counts. Two specimens had normal prevortex platelet counts and increased postvortex platelet counts. Two specimens had increased platelet counts before and after vortexing. In addition, 71 specimens had platelet counts in the normal range before and after vortexing. Of the 71 specimens with results in the normal range, 66 yielded the same or higher platelet counts after vortexting (0-153 × 10^3/μL higher), whereas 5 specimens yielded lower platelet counts after vortexing (–1 to –10 × 10^3/μL lower) The slight decrease in platelet counts for these 5 specimens may be due to normal instrument variability.

TABLE 1 displays breakdowns for all platelet count ranges, and **TABLE 2** displays the breakdown of specimen results by instrument.

TABLE 1. Distribution of platelet count results by range (n = 97)

Platelet count range (×10^3/μL)	No. (%)[a]	Change range (× 10^3/μL)
Initial flagged platelet count <140 and postvortex platelet count <140	11 (11)	1 specimen: –5 10 specimens: 0-23
Initial flagged platelet count <140 and postvortex platelet count >140	11 (11)	11 specimens: 11-87
Initial flagged platelet count = 140-400 and postvortex platelet count = 140-400	71 (73)	5 specimens: –10 to –1 66 specimens: 0-153
Initial platelet count = 140-400 and postvortex platelet count >400	2 (2)	2 specimens: 80-116
Initial flagged platelet count >400 and postvortex platelet count >400	4 (4)	1 specimen: –17 3 specimens: 5-116

[a]*Percentages may not total 100 because of rounding.*

TABLE 2. Distribution of platelet count results by instrument

Instrument designation code	No. of specimens	Change range	*r* value
1	33	0-92	0.957
2	19	–7 to 153	0.904
3	24	–17 to 127	0.938
4	21	–1 to 127	0.844

Only one of the 97 specimens (1%) was flagged for other abnormalities and required a smear review for reasons other than platelet clumps.

Pearson *r* analysis was performed using data from all 97 specimens to determine the correlation of results between pre- and postvortexing parameters for each of the following instrument-measured parameters: WBC count, RBC count, Hb, Hct, and platelet count. All Pearson *r* scores were >0.9 (**TABLE 3**). This finding demonstrates no significant variation between the pre- and postvortex results for these parameters. **FIGURE 1** graphically demonstrates the correlation between results of pre- and postvortex platelet counts. **FIGURE 2** shows pre- and postvortex platelet counts for each specimen on separate lines.

Discussion

Processes for dealing with platelet count flags in the medical laboratory vary among laboratories. Anecdotally, some laboratories require re-collection of specimens yielding platelet clump flags based solely on the presence of the flags. Others perform a smear review and attach a comment regarding the presence or absence of platelet clumps to the report with the instrument-generated platelet count, or replacing the count with an estimate of adequate, decreased, or increased. Still others claim that vortexing the specimen and rerunning the specimen are acceptable practices. In my literature review, I found no studies that addressed best practices for handling of specimens with platelet clump flags not caused by clotting or EDTA-induced clumping. A survey of laboratory practices regarding platelet clumping may prove to be revealing.

This study was performed to validate the use of vortexing specimens that have triggered platelet clump flags that would have otherwise had normal results and had results automatically filed into patient medical

TABLE 3. Pearson *r* value: CBC parameters before and after vortexing (n = 97)

Parameter	*r* value
White blood cell count	0.998
Red blood cell count	0.919
Hemoglobin	0.997
Hematocrit	0.995
Platelet count	0.929

FIGURE 1. Correlation of platelet-count results: initial results vs postvortexing results (n = 97; *r* = 0.929).

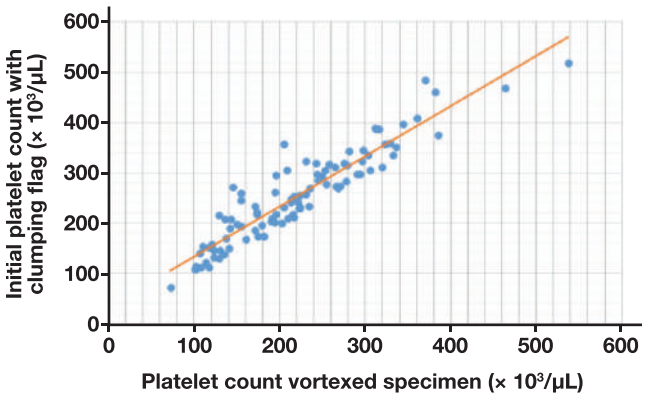

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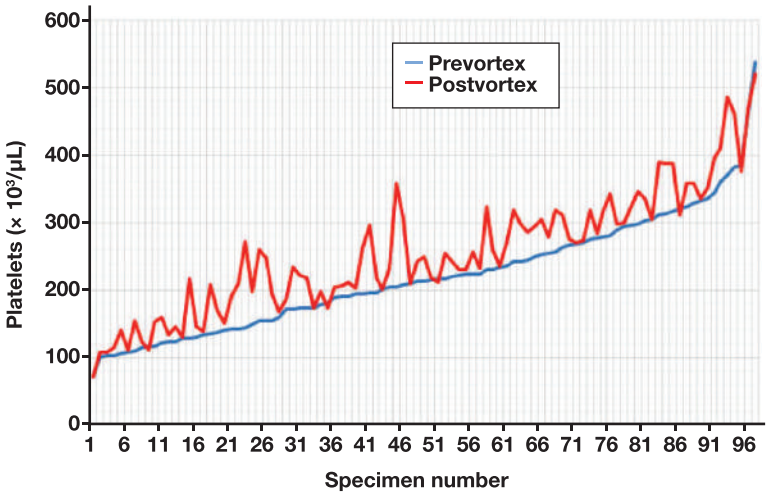
FIGURE 2. **Comparison of prevortex platelet counts with postvortex platelet counts.**



records, therefore improving turnaround times and eliminating the need for patient specimen re-collection. Specimens exhibiting other flags, such as RBC morphology or WBC differential flags, should be reviewed even if platelet clump flags are resolved.

The results of this study show that vortexing specimens that yield platelet clump flags when the platelet counts are within normal reference ranges, especially when no other abnormalities are present, may prove to be effective in processing these specimens. Also, data from specimens with low (<145,000) or high (>445,000) platelet counts that demonstrate correction by an additional round of vortexing may support the use of vortexing to disaggregate clumped platelets in these specimens. However, the number of those specimens encountered during this study (n = 26) is too low to make this assertion statistically viable.

Conclusion

Platelet clump flags are not always reliable and can result in specimen re-collection, creating additional patient safety concerns and generating addition costs to the facility. Vortexing and rerunning these specimens may provide a solution to unnecessarily re-collecting specimens flagged for platelet clumping, especially those specimens with normal CBC parameters. Each laboratory needs to determine a process for handling hematology analyzer–generated platelet clump flags and to include it in their protocol documentation. Validation of the process of vortexing specimens should identify the vortex setting and length of time that specimens are to be vortexed. Also, the protocol should include at what point in the process vortexing may occur, such as after smear preparation. If the platelet clump flag is not resolved, alternative processes, including re-collection, may be indicated.

Conflict of Interest Disclosure

The author has nothing to disclose.

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Are tube fill volumes below 90% a rejection criterion for all coagulation tests?

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Key words: coagulation tests, underfilled tube, total allowable error, reference change value

Abbreviations: PT, prothrombin time; aPTT, activated partial thromboplastin time; TAE, total allowable error; RCV, reference change value; CVa, analytical variation; CVi, biological variation

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ABSTRACT

Background: Rejected samples lead to prolonged turnaround time and delayed diagnosis and treatment of patients. This study was conducted to determine minimum acceptable sample volume in Sarstedt brand coagulation tubes to reduce high sample rejection rate.

Methods: Blood samples were drawn from 20 participants (10 healthy volunteers and 10 patients receiving oral anticoagulant) into coagulation tubes. Six samples were taken from each participant, with tube fill volumes of 100%, 90%, 80%, 70%, 60%, and 50%. Prothrombin time (PT), active partial thromboplastin time (aPTT), and fibrinogen tests were analyzed.

Results: According to quality performance specifications, the tube fill volume must be at least 70% for PT and aPTT and 50% for fibrinogen. There was no statistical difference in samples from healthy volunteers for PT, aPTT, and fibrinogen tests when the minimum tube fill volume was at least 80%, 90%, and 50%, respectively. These percentages were 50%, 70%, and 60%, respectively, in patients receiving oral anticoagulant.

Conclusions: Sarstedt tubes meet quality standard specifications at a 70% fill rate for PT and aPTT and a 50% fill rate for fibrinogen. Comprehensive studies with larger populations are needed to accept these values as sample acceptance criteria for the laboratory.

Introduction

Clinical decision-making is largely (70%) dependent on laboratory test results and therefore it is necessary to ensure the reliability and accuracy of laboratory results.[1] Most laboratory errors occur at the preanalytical stage (46%-68%).[2] A study in Italy reported that 62% of laboratory errors occur before the sample reaches the laboratory.[3] Although guidelines and standard protocols exist for blood sampling, compliance is low.[4]

Laboratory errors cause an increase in health costs and a decrease in patient satisfaction as well as a misjudgment of patients in the clinical decision stage. A study of a 650-bed hospital in the US reported that the costs of preanalytical errors accounted for 0.23% to 1.2% of the total hospital expenditure of approximately $1,199,122. For these reasons, a standardized, reliable test result is the goal of quality assurance in the laboratory.[5]

Many preanalytical variables can affect routine coagulation test results. Sodium citrate concentration, blood-to-liquid anticoagulant ratio, insufficient tube fill, and clotted sample may alter activated partial thromboplastin time (aPTT), prothrombin time (PT), and fibrinogen test results. Insufficient filling of citrate tubes reduces the blood-citrate ratio, which should be 9:1.[6] Coagulation tests may be prolonged due to the dilution effect of the citrate volume and the reduced availability of calcium in the assay reaction. The Clinical & Laboratory Standards Institute (CLSI) H21-A5 recommends that citrate tubes be at least 90% full for coagulation tests.[7] Some publications have shown that a fill volume of between 60% and 90% is acceptable, depending on the test and reagent type, citrate concentration, total tube volume, and tube brand.[8-10] Unfortunately, there is no study in the literature about the minimum fill volume for the tube brand (Sarstedt) we use in our laboratory.

The need for reliable results in clinical laboratories must be weighed against the disadvantage of a rejected sample for patients and health care providers. Therefore, clinical laboratory professionals should establish a policy for the minimum acceptable sample volume for each brand in standard tubes containing citrate anticoagulant for routine coagulation testing, and laboratories should validate these findings in their own settings. In this study, we validated the minimum tube fill volume of citrated tubes and included different clinical and analytical acceptance criteria.

Methods

Informed consent was obtained before venous blood samples were taken from the antecubital area. Ethics approval was obtained from the local health practices and research center clinical research ethics committee

(No. 3639, 23/08/2022), and the study was conducted in accordance with the Declaration of Helsinki.

Venous blood was drawn from 20 subjects (10 healthy volunteers and 10 patients receiving oral vitamin K antagonist anticoagulant therapy) with a syringe system and then immediately transferred without any order into 3.6 mL plastic vacuum tubes containing 3.2% buffered sodium citrate (Sarstedt). A tube filled to the fill line (2.7 mL blood + 0.3 mL anticoagulant) was considered 100% full. Blood in the amounts of 2.7 mL, 2.4 mL, 2.1 mL, 1.8 mL, 1.5 mL, and 1.2 mL was transferred to tubes to obtain tube fill rates of 100%, 90%, 80%, 70%, 60%, and 50%, respectively. The filled tubes were mixed by slowly inverting them from end to end. Six citrate samples were taken from each participant, with tube fill volumes of 100%, 90%, 80%, 70%, 60%, and 50%. International normalized PT, aPTT, and fibrinogen tests were determined on a Stago Compact Max 3 coagulation analyzer. All assays were performed with Sta-Neoptimal, STA-C.K. Prest, and STA-Liquid Fib reagents, respectively. Samples were sequentially analyzed immediately after centrifugation (10 minutes, 2000*g*, room temperature). For each underfilled tube, the absolute percent difference was calculated by the formula

$$\text{PT, s (percent difference)} = [\text{PT, s (full tube)} - \text{PT, s (underfilled tube)}] / \text{PT, s (full tube)} * 100.$$

Statistical analyses were performed with IBM SPSS (IBM SPSS Statistics for Windows, 2015, version 23.0). The Shapiro-Wilk test was used to determine whether the data showed a normal distribution. The results are presented as mean ± standard deviation, median (minimum-maximum), or frequency and percentage. Nonnormally distributed data was compared with Mann-Whitney *U* tests with $P < .05$ considered to be significant.

Total allowable error (TAE) and reference change value (RCV) or critical difference (CD) were also used to evaluate the result.[11,12] Analytical variation (CVa) was calculated based on our low/high level internal quality control result, and within-subject biological variation (CVi) was obtained from the study of Ricós et al.[11] We calculated RCV according to the formula $2^{(1/2)} \times Z \times (CVa^2 + CVi^2)^{(1/2)}$ (**TABLE 1**).

TABLE 1. Quality performance specifications for PT, aPTT, and fibrinogen tests

Test	CVa (%) N/H level	CVi (%)	RCV (%) N/H level	TAE (%)
PT	5.4/7.2	4.0	18.6/22.8	15
aPTT	3.6/3.8	2.7	12.5/12.9	15
Fibrinogen	3.0/3.8	10.7	30.7/31.5	20

aPTT, activated partial thromboplastin time; CVa, analytical variation; CVi, intraindividual biological variation; N/H, normal/high level; PT, prothrombin time; RCV, reference change value; TAE, total allowable error.

Results

By evaluating the data obtained from the entire group of 20 people, the absolute percentage difference of insufficiently filled tubes was calculated (**TABLE 2**). **TABLES 3** and **4** show the absolute percentage difference of insufficiently filled tubes of participants who are healthy and taking oral anticoagulants, respectively.

According to the minimum quality performance specifications (both RCV and TAE), the tube fill rate must be at least 70% for PT and aPTT tests. For fibrinogen, even 50%, the last rate we tested, meets quality performance specifications (**TABLES 5** and **6** and **FIGURE 1**).

Discussion

Improper sample collection is common in laboratory practice, and these samples should be rejected, as the reliability of test results can be compromised. In line with the CLSI H21-A5 recommendation of a 90% minimum tube fill volume for coagulation tests, the most common reason for rejection of coagulation tubes in our laboratory is insufficient sample. This rate is even higher in emergency rooms, where blood collection personnel change frequently and are very crowded and busy from time to time. Similarly, in a study conducted in a tertiary hospital, the most common preanalytical error was found to be insufficient sampling (54%).[13] Sample rejection causes delayed analysis of patient tests, delay in patient diagnosis, loss of manpower, time, and greater cost. The need for reliable results must be weighed against the disadvantage of a rejected sample for patients and health care providers.[14] For these reasons, we conducted this study to determine the minimum acceptable sample volume in the Sarstedt brand primary coagulation tubes we currently use to reduce the high sample rejection rate.

In this study, statistical difference was observed when the minimum tube fill volume for PT was 70% in tubes taken from healthy individuals ($P = .014$). However, even at 50% of the minimum tube fill volume, there was no significant difference from patients receiving oral anticoagulant therapy. According to quality performance specifications (for both RCV and TAE), minimum tube fill rate for PT should be at least 70%. For the first time, Peterson and Gottfried[15] explained that underfilling of citrated tubes creates a significant bias, whereas overfilling rarely makes test results unreliable. Reneke et al[9] reported that accurate PT values were obtained when Sherwood Medical–branded blood tubes containing sodium citrate were filled up to 65% of the nominal volume for healthy subjects and 90% for patients receiving oral anticoagulant therapy, respectively. Adcock et al[8] observed no significant difference in samples filled between 60% and 100% for PT with Becton Dickinson–branded tubes. Lippi et al[16] It showed that the citrate tube (Terumo Europe) drawn at 67% for PT is still reliable. Ver Elst et al[6] validated the minimal citrate tube fill volumes of 73% for PT. Although a 70% filling rate for PT

TABLE 2. Absolute percent difference of underfilled tubes of all participants

	100%	90%	80%	70%	60%	50%
PT, s mean ± SD (% difference)	23.0 ± 13.0	23.8 ± 13.6 (3.3)	25.0 ± 14.3 (8.8)	26.3 ± 15.3 (14.2)	28.8 ± 17.4 (25.1)	37.5 ± 31.8 (62.8)
aPTT, s mean ± SD (% difference)	30.0 ± 4.6	30.8 ± 4.8 (2.6)	31.8 ± 5.0 (5.9)	32.9 ± 5.5 (9.7)	35.5 ± 7.3 18.4)	39.0 ± 9.9 (30.1)
Fibrinogen, g/L mean ± SD (% difference)	3.8 ± 1.1	3.8 ± 1.1 (1.3)	3.6 ± 1.0 (4.9)	3.5 ± 1.0 (8.4)	3.4 ± 1.0 (10.3)	3.2 ± 0.9 (15.2)

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

TABLE 3. Absolute percent difference of underfilled tubes of healthy participants

	100%	90%	80%	70%	60%	50%
PT, s mean ± SD (% difference)	12.7 ± 1.0	13.1 ± 1.1 (2.8)	13.6 ± 0.8 (6.9)	14.1 ± 1.2 (10.7)	14.9 ± 1.3 (17.4)	16.6 ± 2.0 (30.4)
aPTT, s mean ± SD (% difference)	27.0 ± 1.4	27.4 ± 1.4 (1.4)	28.1 ± 1.3 (4.1)	28.9 ± 1.9 (7.2)	30.3 ± 1.6 (12.3)	32.5 ± 2.3 (20.2)
Fibrinogen, g/L mean ± SD (% difference)	3.4 ± 0.8	3.3 ± 0.8 (1.4)	3.2 ± 0.7 (4.6)	3.1 ± 0.7 (8.9)	3.0 ± 0.7 (10.2)	2.9 ± 0.7 (14.9)

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

TABLE 4. Absolute percent difference of underfilled tubes of participants taking oral anticoagulant

	100%	90%	80%	70%	60%	50%
PT, s mean ± SD (% difference)	33.2 ± 11.2	34.5 ± 11.7 (3.8)	36.5 ± 11.8 (9.8)	38.5 ± 12.7 (15.8)	42.6 ± 14.5 (28.3)	58.4 ± 34.1 (75.8)
aPTT, s mean ± SD (% difference)	33.1 ± 4.6	34.2 ± 4.6 (3.2)	35.4 ± 4.5 (7.0)	36.9 ± 5.0 (7.2)	30.3 ± 1.6 (11.5)	45.6 ± 10.4 (37.7)
Fibrinogen, g/L mean ± SD (% difference)	4.2 ± 1.3	4.1 ± 1.2 (1.2)	4.0 ± 1.2 (5.1)	3.9 ± 1.1 (8.0)	3.8 ± 1.1 (10.4)	3.5 ± 1.1 (15.5)

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

TABLE 5. Influence of underfilling citrate tubes on the results of PT, aPTT, and fibrinogen tests of healthy participants[a]

Tube fill ratio	PT, s median (minimum-maximum)	*P*	aPTT, s median (minimum-maximum)	*P*	Fibrinogen, g/L median (minimum-maximum)	*P*
100%	12.3 (11.6-14.4)	>.05	27.1 (24.9-29)	>.05	3.2 (2.07-5.0)	>.05
90%	12.6 (11.3-14.9)	>.05	27.5 (25.4-29.6)	>.05	3.1 (2.0-5.0)	>.05
80%	13.5 (12.4-15.1)	>.05	29.4 (25.9-31.6)	**.019**	3.1 (2.0-4.7)	>.05
70%	13.6 (12.5-16.4)	**.014**	34.8 (27.8-42.4)	**.001**	2.9 (1.9-4.5)	>.05
60%	14.4 (13.4-17.2)	**.002**	31.1 (27.8-32.0)	**.001**	2.8 (1.8-4.5)	>.05
50%	16.0 (14.1-20.2)	**<.001**	32.3 (28.6-35.7)	**<.001**	2.7 (1.6-4.3)	>.05

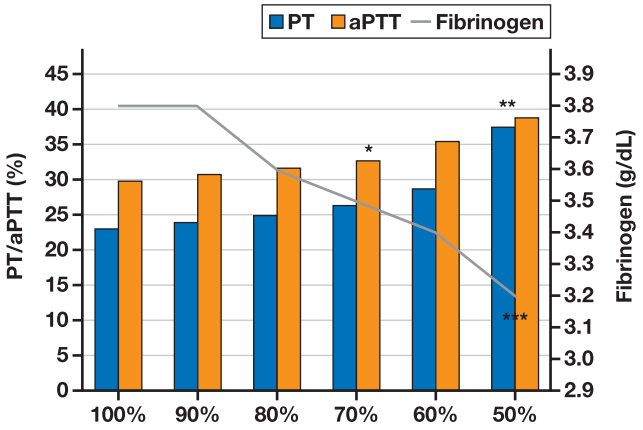
aPTT, activated partial thromboplastin time; PT, prothrombin time.
[a]*Bold denotes statistical significance.*

TABLE 6. Influence of underfilling citrate tubes on the results of PT, aPTT, and fibrinogen tests of participants taking an oral anticoagulant[a]

Tube fill ratio	PT, s median (minimum-maximum)	*P*	aPTT, s median (minimum-maximum)	*P*	Fibrinogen, g/L median (minimum-maximum)	*P*
100%	32.1 (18.9-55.9)	>.05	33.3 (26.4-41.6)	>.05	3.8 (3.1-7.5)	>.05
90%	33.3 (19.0-57.4)	>.05	34.8 (27.8-42.4)	>.05	3.7 (3.1-7.3)	>.05
80%	33.3 (19-57.4)	>.05	33.9 (28.9-43.0)	>.05	3.6 (3.1-7.0)	>.05
70%	37.6 (20.0-59.5)	>.05	37.8 (29.0-45.7)	>.05	3.5 (3.0-6.6)	>.05
60%	38.5 (20.9-64.7)	>.05	40.9 (30.7-51.2)	**.019**	3.3 (2.8-6.6)	>.05
50%	41.6 (21.8-69.2)	>.05	44.4 (32.2-67.1)	**.004**	3.2 (2.5-6.4)	**.02**

aPTT, activated partial thromboplastin time; PT, prothrombin time.
[a]*Bold denotes statistical significance.*

FIGURE 1. Influence of underfilled tubes on the results of prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen tests of all participants. *There is a significant difference in the aPTT value when the minimum tube fill volume is 70% or less (P = .03). **There is a statistically significant difference in the PT value when the minimum tube fill volume is 50% or less (P = .029). ***There is a statistically significant difference in the fibrinogen value when the minimum tube fill volume is 50% or less (P = .011).



creates a significant difference for healthy participants, at least a 70% filling rate for PT may be acceptable because it does not create a clinically significant difference for the patient (since it does not exceed RCV and TAE). However, studies with a larger sample volume and a larger population are needed to implement the rule of rejecting only samples below 70% for PT in our laboratory.

Similar to other studies, the aPTT was more sensitive to underfills and there was no significant difference when the minimum filling volume was at least 90% in healthy subjects and at least 70% in those using oral anticoagulants. For the first time, Reneke et al, in their study with Sherwood Medical tube brand, found that APTT was much less tolerant to underfilling, with prolonged values observed in most specimens filled to less than 90% of capacity.[9] Lippi et al showed that a clinically significant difference was observed when the citrated tube (Terumo Europe) was withdrawn less than 89% for aPTT.[16] Ver Elst et al[6] confirmed the 90% minimum citrate tube fill volumes for aPTT. However, Adcock et al[8] observed no significant difference in samples filled between 70% to 100% for aPTT with Becton Dickinson–branded tubes. Similarly, aPTT results were reliable when the tube fill volume was at least 70%, according to quality specification (for both RCV and TAE), in this study. Similar to PT, studies with larger sample volumes and larger populations are needed so that we can apply the rule of rejecting samples below 70% for aPTT.

For fibrinogen, no significant difference was observed in healthy individuals, even when the tube fill volume was 50%, whereas there was no significant difference up to 60% in those receiving anticoagulant therapy. Considering the minimum quality performance characteristics, the minimum tube fill rate for fibrinogen should be 50% and above. Lippi et al[16] stated that a clinically significant difference was observed when the tube was filled less than 78% for fibrinogen. In another study, they showed that 63% tube fill volume was sufficient for fibrinogen.[6] In our study, we found that a lower fill volume was sufficient for fibrinogen compared with the literature.

The limitations of this study are the low number of participants and the inclusion of patients using only one type of oral anticoagulant. A new study should be conducted when the tube brand is changed, as these findings may differ.

In conclusion, rejected sample leads to new sample requests, prolonging turnaround time and delaying the diagnosis and treatment of critically ill patients.[17] Unfortunately, it is not easy to standardize all preanalytical processes and insufficient sample error is very common in citrated coagulation tubes.[18] Although the filling rate of 70% for PT and aPTT and 50% for fibrinogen in the Sarstedt brand coagulation tubes we use does not make a significant difference in terms of clinical decision, a larger study is needed to confirm these rates as sample acceptance criteria.

Acknowledgments

We thank the personnel who helped with sampling.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Whole-exome sequencing uncovers a novel *EFEMP2* gene variant (c.C247T) associated with dominant nonsyndromic thoracic aortic aneurysm

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Key words: whole-exome sequencing, thoracic aortic aneurysm, pathogenic, variant, genetic, *EFEMP2*

Abbreviations: TAA, thoracic aortic aneurysm; WES, whole-exome sequencing; CT, computed tomography; ATAA, ascending TAA; ACMG, American College of Medical Genetics and Genomics; PCR, polymerase chain reaction; TGFβ, transforming growth factor β; LTBPs, latent TGFβ binding proteins; LOX, lysyl oxidase; LLC, large latent TGFβ complex; LAP, latency-associated propeptide; SLC, small latent complex; SOV, sinus of Valsalva; RMSD, root mean square deviation

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ABSTRACT

Background: Thoracic aortic aneurysm (TAA) is a multifactorial disorder. Familial TAA, which is more clinically aggressive, is associated with a high risk of lethal dissection or rupture. Genetic evaluation can provide TAA patients with personalized treatment and help in predicting risk to family members.

Objective: The purpose of this investigation was to report a likely pathogenic variant in the *EFEMP2* gene that may contribute to TAA in a family with a documented history of the condition.

Methods: In the index patient, the causative genetic predisposition was identified using whole-exome sequencing. The potential likely pathogenic effect of the candidate variant was further analyzed through bioinformatics analysis, homology modeling, and molecular docking.

Results: The results revealed a likely pathogenic heterozygous variant, c.247C>T p.Arg83Cys, in exon 4 of the *EFEMP2* gene (NM_016938), which was predicted to have disease-causing effects by MutationTaster, PROVEAN, SIFT, and CADD (phred score = 27.6).

Conclusion: In this study, a likely pathogenic variant in the *EFEMP2* gene was identified in an Iranian family with a dominant pattern of autosomal inheritance of TAA. This finding underscores the importance of conducting molecular genetic evaluations in families with nonsyndromic TAA and the significance of early detection of at-risk family members.

Introduction

Thoracic aortic aneurysm (TAA) refers to the enlargement of the aorta to at least 50% greater than its expected diameter for an individual of the same age and sex. TAA can be classified based on its anatomical location, with the majority (60%) occurring in the ascending aorta, 10% in the aortic arch, and 30% in the descending aorta.[1] The incidence of TAA is estimated to be 6 to 10 per 100,000 people annually.[2] TAA is a multifactorial disorder that results from a combination of genetic, environmental, and physiological factors.[3] Familial TAA, which occurs in 20% of cases, is more clinically aggressive and presents at a younger age with a faster rate of enlargement than TAA without a family history; thus, early intervention may be crucial in these cases.[4,5] Familial TAA often involves the ascending aorta and includes conditions such as Marfan, Loeys-Dietz, and vascular Ehlers-Danlos syndromes[6] as well as nonsyndromic TAA, which is limited to the cardiovascular system.[4,6,7] Several genes have been linked to TAA, including *FBN1*, *FBN2*, *COL3A1*, *EFEMP2*, *MFAP5*, *TGFβR1*, *TGFβR2*, *SMAD3*, *TGFβ2*, *TGFβ3*, *SKI*, *SLC2A10*, *SMAD4*, *ACTA2*, *MYH11*, *MYLK*, *PRKG1*, *MAT2A*, and *FLNA*.[8,9] TAA with dissection is a critical condition that is associated with significant morbidity and mortality.[10,11] The type and location of the aortic involvement and the underlying gene variant in patients with familial TAA affect the growth rate and clinical phenotype, which can predict prognosis and response to therapy[12]; therefore, genetic investigation of patients with TAA is important for personalized treatment options and risk prediction for family members. In this study, we report a likely pathogenic variant in the *EFEMP2* gene in a family with documented ascending aortic aneurysm. To our knowledge, this is the first time that a dominant pattern manifestation of *EFEMP2* has been detected using whole-exome sequencing (WES).

Methods

Family Recruitment and Clinical Characteristics

A 65-year-old woman who was asymptomatic was referred to our cardiovascular tertiary care center for screening of ascending thoracic aortic aneurysm (ATAA) due to her positive family history (**FIGURE 1A**: II-6). The patient had no history of any diseases or medication use and came from a family of 8, including 2 brothers and 3 sisters (**FIGURE 1A**). Her mother had died suddenly at 68 years of age without any prior symptoms (**FIGURE 1A**: I-2). Additionally, 2 of the patient's siblings had died from acute aortic dissection (**FIGURE 1A**: II-1, II-3). The remaining siblings (2 female and 1 male, aged 71, 69, and 65 years, respectively) of the family were evaluated by a cardiologist and underwent echocardiography, which revealed the presence of ATAA in 1 sibling, who underwent urgent surgery due to the enlargement of the ascending aorta and severe aortic valve regurgitation (**FIGURE 1A**: II-5). The other pedigree members were healthy. Deceased individuals had been diagnosed with TAA before their death. I-2 died at age 60, II-1 at age 52, and II-3 at age 58. Our case study individual (**FIGURE 1A**: II-6) was evaluated by a cardiologist and a geneticist using echocardiography and aortic computed tomography (CT) angiography. A thorough medical history investigation was also conducted, and a peripheral blood sample was collected for genetic evaluation using WES. The study was approved by the Ethics Committees of Rajaie Cardiovascular Medical and Research Center, Iran University of Medical Sciences, Tehran, Iran (IR.RHC.REC.1400.005) and was conducted in compliance with the Declaration of Helsinki. Written informed consent was obtained from the patient.

Whole-Exome Sequencing

The extraction of DNA was carried out using an in-house salting out method. The wet lab of WES was performed on the proband (**FIGURE 1A**: II-6) by Macrogen and the raw data (fastq file) was analyzed by the Cardiogenetic Research Center at the Rajaie Cardiovascular Medical and Research Center, Iran University of Medical Sciences, Tehran, Iran. The library preparation was carried out according to the manufacturer's protocol using the Illumina NovaSeq6000 platform (Illumina) for sequencing the paired-end reads. The reference genome (GRCh37/hg19) was used for the alignment of the reads using the Burrows-Wheeler Aligner (BWA) algorithm (version 0.7.12). Variants were identified and annotated using the Genome Analysis Toolkit (GATK, version 4.1.4.1) and ANNOVAR, respectively. The pathogenicity of the variants was evaluated using CADD, SIFT, PolyPhen-2, PROVEAN, and MutationTaster. Furthermore, the variants were classified according to the guidelines set by the American College of Medical Genetics and Genomics (ACMG).[13]

Variant Validation

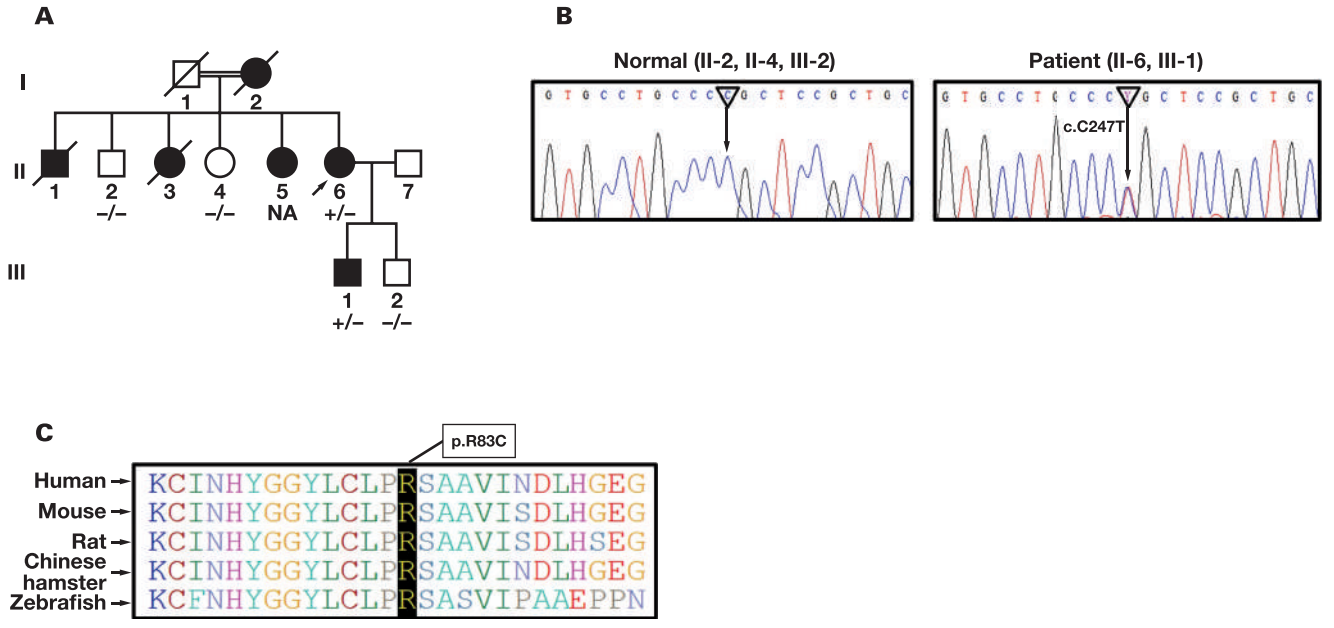
To confirm and segregate any variants detected in the proband's DNA, polymerase chain reaction (PCR) and direct Sanger sequencing were performed on the proband and other family members. The PCR was carried out using specific primers (forward primer: ATGAGGGCAACAGTGGGA, reverse primer: AGGCTGGTGAACTCTTACGC) for the candidate variant on a SimpliAmp thermal cycler (Thermo Fisher Scientific). The PCR products were analyzed using an ABI Sequencer 3500XL PE (Applied Biosystems) (**FIGURE 1B**).

Homology Modeling and Docking

Fibulin-4 and TGFβ Signaling

The *EFEMP2* gene encodes the extracellular matrix protein fibulin-4, which plays a crucial role in elastic fiber assembly and is highly expressed in the medial layer of the aorta and contributes to aortic contractility.[9]

FIGURE 1. Information regarding the family with thoracic aortic aneurysm (TAA). A, Pedigree of the family with TAA. B, Sequence chromatograms of the likely pathogenic heterozygous variant *EFEMP2* c.C247T in the proband (II-6) and her son (III-I). C, Amino acid evolutionary conservation analysis by CLUSTALW (https://www.genome.jp/tools-bin/clustalw). The recognized mutated site of (EFEMP2 p.Arg83Cys) is greatly conserved among species.



A decrease in fibulin-4 levels in the extracellular matrix can lead to altered interactions with binding partners, such as latent transforming growth factor β (TGFβ) binding proteins (LTBPs), fibrillin-1, and lysyl oxidase (LOX), and subsequently, to impaired elastogenesis. Fibulin-4 seems to be a mediator of the association between LTBP-1 and/or the large latent TGFβ complex (LLC) and microfibrils. When fibulin-4 is impaired, LTBP is no longer able to bind the elastic fibers, and LTBP and LLC are released, making TGFβ more prone to activation.[14] Transforming growth factor β is secreted from cells and is bound noncovalently with latency-associated propeptide (LAP) as a dimeric small latent complex (SLC) or as a large LLC comprising SLC bound covalently to an LTBP. Only LTBP-1 and -3 bind TGFβ strongly. LTBPs are structurally related to fibrillins.[15,16] Fibulin-4 binds to the N-terminal region of fibrillin-1 and the N-terminal half of LTBP1 with high affinity, and can simultaneously bind LTBP-1 and fibrillin-1.[17] This implicates fibulin-4 as a key mediator in the association of LTBP-1 with microfibrils, a process that is important in regulating the bioavailability of TGFβ for activation and signaling.[14,17] Additionally, fibulin-4 binds to LOX, which is important for elastic fiber assembly and also represses TGFβ.[18] Studies of aortic tissue from humans with fibulin-4 deficiency have shown increased TGFβ signaling, which may be due to impaired LOX-mediated TGFβ repression.[19] TAA disease has been linked to an increase in TGFβ signaling,[9] and fibulin-4 deficiency has been linked to dysregulation and enhancement of TGFβ signaling in the aorta.[20] In the current study, to investigate the effect of the p.Arg83Cys variant of fibulin-4 on the TGFβ signaling pathway, fibulin-4 protein (normal and p.Arg83Cys variant) was docked with fibrillin-1 and LOX.

Protein-Protein Docking

To conduct computer-assisted molecular docking studies, the 3D structures of fibulin-4 (p.Arg83Cys variant) and the N-terminal domain of fibrillin-1 were predicted using AlphaFold2 and the MMseqs2 server.[21] The 3D structures of normal fibulin-4 and LOX were obtained from the UniProtKB database (https://www.uniprot.org/). Hydrogen atoms were added to all of these structures using ViewerLite v.5. The energy

FIGURE 2. A-C, Aortic computed tomography angiography revealed an ascending aorta aneurysm and sinus of Valsalva without indications of dissection. D, An ascending aorta aneurysm and sinus of Valsalva were discovered on long axis view echocardiography. 1, ascending aorta aneurysm; 2, sinus of Valsalva.

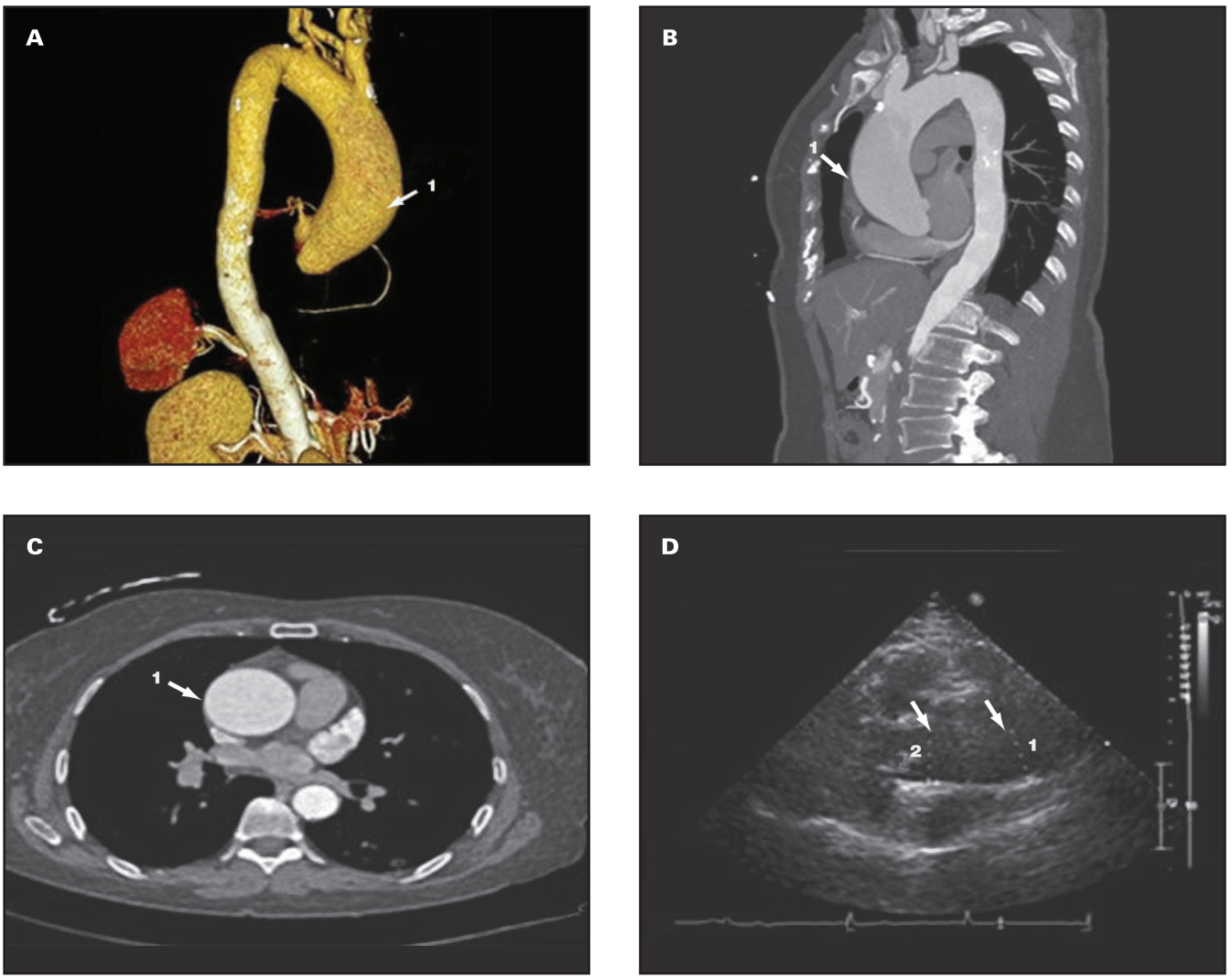
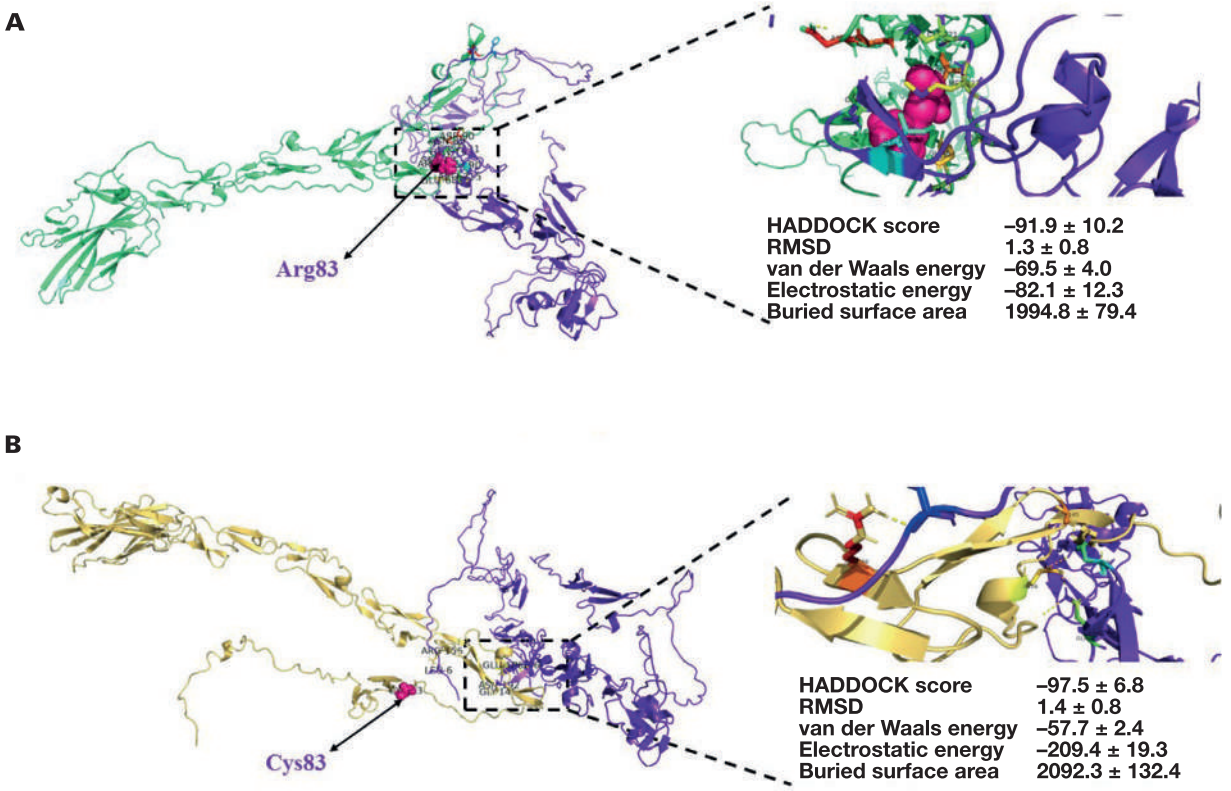


FIGURE 3. **Visual representation of protein interactions between fibulin-4 and the N-terminal domain of fibrillin-1. A, The interaction between the normal fibulin-4 protein (green) and the N-terminal domain of fibrillin-1 protein (purple). B, The interaction between the variant fibulin-4 protein (yellow) and the N-terminal domain of fibrillin-1 protein (purple), as determined by molecular docking analysis using PyMOL v.2.5.2.**



minimization of the structures was performed using the YASARA Energy Minimization Server (http://www.yasara.org/minimizationserver.htm),[22] and the SCE files from the YASARA web server were then imported into YASARA View v.20.12.24 and saved as PDB files. The molecular docking analysis was conducted through the HADDOCK server (https://wenmr.science.uu.nl/haddock2.4/).[23,24] The details of their interactions were analyzed with the use of PyMOL v.2.5.2 and LigPlus+ v.2.2.4.[25,26]

Results

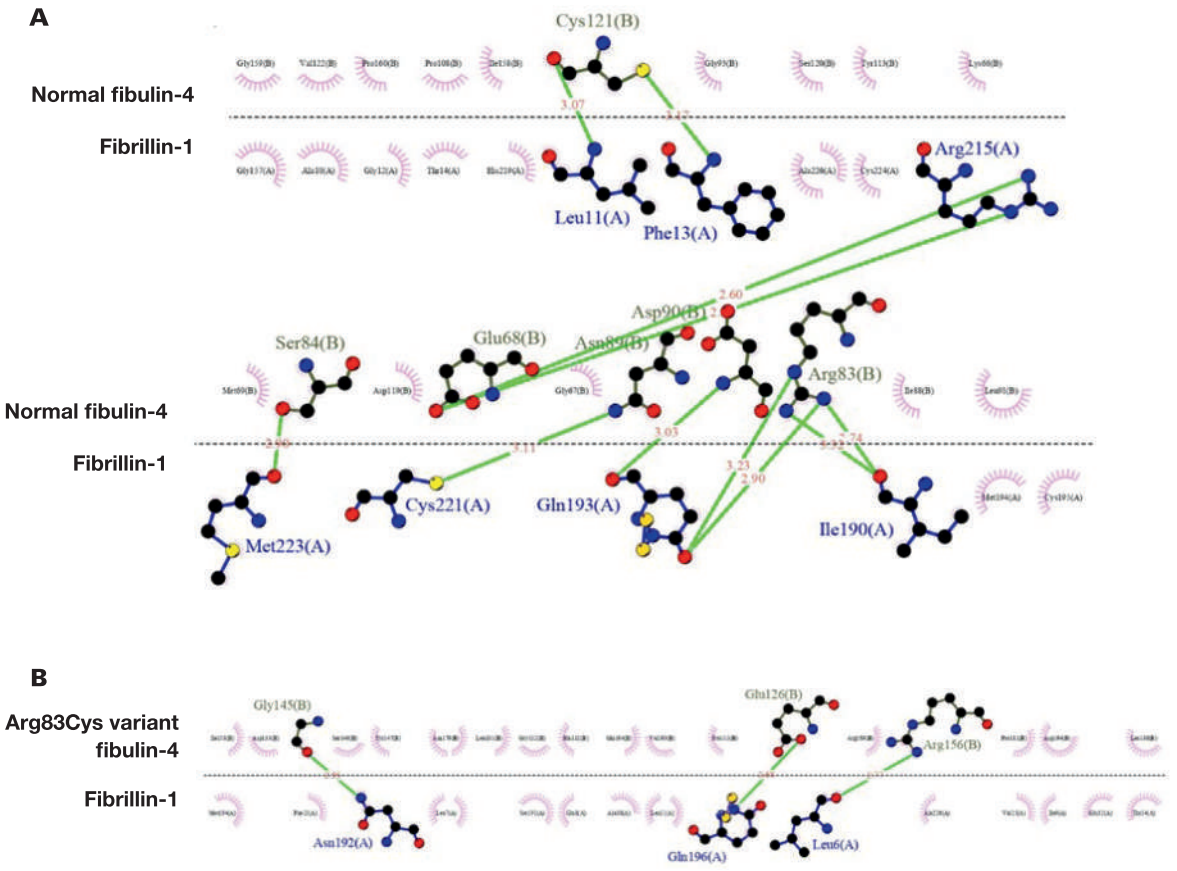
Clinical Analysis

The patient followed up by regularly scheduled imaging evaluations throughout the course of her treatment. In October 2020, CT angiography revealed a maximum diameter of 46.7 mm in the proximal section of the aortic root. In October 2021, an echocardiography showed an aneurysm diameter of 47 mm and a sinus of Valsalva (SOV) of 37 mm (normal diameter of the ascending aorta has been defined as 33.4 mm in men and 30.5 in women). During the next annual follow-up, the aneurysm diameter measured 48 mm and the SOV was 38 mm. In 2022, the patient presented to the emergency department with complaints of atypical chest pain and malaise. To rule out the presence of coronary artery disease, coronary CT angiography was performed, which ultimately revealed mild coronary artery disease. The most recent CT angiography indicated a diameter of 47.5 mm for the patient's ATAA (**FIGURE 2A-D**).

Molecular Analysis

We performed WES for the proband to uncover the genetic basis of the phenotype and establish a clinical diagnosis. The results revealed a likely pathogenic variant, c.247C>T p.Arg83Cys, in exon 4 of the *EFEMP2* gene (NM_016938) with autosomal dominant pattern. Based on ACMG guidelines, this variant has PP1, PP3, PP4, and PM2 criteria. This heterozygous variant was also found in the proband's son (**FIGURE 1B**: III-1) and was predicted to have disease-causing effects by MutationTaster, PROVEAN, and SIFT. The other affected individual (**FIGURE 1B**: II-5) was not available for more investigation. The CADD score for the variant was 27.6 and was not present in any databases, that is, ExAC, GME, esp6500, or Iranome. Due to the alignment analysis, the recognized mutated site of (EFEMP2 p.Arg83Cys) is greatly conserved among species (**FIGURE 1C**). No variants, that is, variants of uncertain significance, pathogenic, and likely pathogenic, were identified in the promoter, UTR and exon-intronic regions boundaries. To further investigate the impact of the variant on protein function, we obtained the best models of human fibulin-4 (p.Arg83Cys variant) and the N-terminal domain of fibrillin-1 from AlphaFold2, with predicted local distance difference test scores of 83.3 and 73.9, respectively. The 3D structures of normal fibulin-4 (ID: AF-O95967-F1) and LOX (ID: AF-P28300-F1) were obtained from the UniProtKB database. The first docking was performed between fibulin-4 (normal/ p.Arg83Cys variant) and the N-terminal domain of fibrillin-1. The results showed a reduced binding affinity for the p.Arg83Cys variant compared to the normal form, as indicated by the HADDOCK score of –91.9 ± 10.2

FIGURE 4. Schematic interaction of the best docking results of normal (A) and variant fibulin-4 (B) with N-terminal domain of fibrillin-1 presented by LigPlus+ v.2.2.4. Hydrogen bonding is in green.



and −97.5 ± 6.8, with root mean square deviation (RMSD) values of 1.3 ± 0.8 Å, 1.4 ± 0.8 Å, respectively. The RMSD has often been used to measure the quality of a binding pose by a computational method, such as docking. Lower RMSD values indicate the most suitable docking pose. The normal fibulin-4 had 11 hydrogen bonds with fibrillin-1, whereas the p.Arg83Cys variant had 3 hydrogen bonds (**FIGURE 3A** and **3B**). The interaction analysis revealed that the normal fibulin-4 interacts with the N-terminal domain of fibrillin-1 through residues Glu68, Cys121, Ser84, Asn89, Asp90, and Arg83, with Arg83, forming 4 strong hydrogen bonds. However, the residues involved in the interaction in the p.Arg83Cys variant model only included Glu126, Arg156, and Gly145 (**FIGURE 4A** and **4B**). This suggests that the p.Arg83Cys variation in fibulin-4 reduces the affinity and the number of hydrogen bonds with the N-terminal domain of fibrillin-1.

The second docking experiment between fibulin-4 (normal/p.Arg83Cys variant) and LOX also showed a reduced binding affinity for the p.Arg83Cys variant. Although Arg83 did not participate in the formation of the link in the normal fibulin-4 form with LOX, the HADDOCK score of normal/mutant fibulin-4 with LOX was −87.9 ± 11.2 and −72.8 ± 7.1, with RMSD values of 1.5 ± 1.0 Å and 2.9 ± 0.9 Å, respectively (**FIGURE 5A** and **5B**). The normal fibulin-4 had 7 hydrogen bonds with LOX, whereas the p.Arg83Cys variant had only 3 hydrogen bonds. The residues involved in the interaction in the normal fibulin-4 form included Tyr113, Glu94, His105, Pro106, Ser146, and Gly145, and in the p.Arg83Cys variant, included Arg156, His105, and Lys157 (**FIGURE 6A** and **6B**). This suggests that the p.Arg83Cys variation in fibulin-4 reduces the affinity with LOX and changes the conformation of fibulin-4 in its interaction with LOX.

Discussion

The discovery of a likely pathogenic *EFEMP2* variant in a patient with autosomal dominant ATAA represents a significant advancement in the field. This report constitutes the first documented case of dominant *EFEMP2* to our knowledge.

Unlike previous cases, the patient in the present study exhibited only vascular involvement, with no signs of cutis laxa skin features.[14,27-29] Similar to the ATAA reported by Al-Hassnan et al,[30] the manifestation of ATAA in our studied pedigree displayed variable symptoms across different age groups, including cyanosis, shortness of breath in infancy, sweating, and carriers without symptoms who were identified through screening. The *EFEMP2* variant c.247C>T p.Arg83Cys identified in the ATAA patient is strongly implicated as the cause of the familial disease. A clear association was established between the variant and the phenotype. The alignment of the EFEMP2 protein sequence highlights the high level of conservation of the arginine residue at codon 83 among species.

The identification of the potentially causative genetic variant in this inherited ATAA has several important implications. Determining the molecular mechanism defect in a proband will facilitate the recognition of a genetic disease. Regular follow-up of presymptomatic individuals who have been genetically established is crucial for effective

FIGURE 5. **Molecular docking analysis for normal and variant fibulin-4 protein with LOX protein by using PyMOL v.2.5.2. A, Protein-protein interaction between normal fibulin-4 and LOX protein (normal fibulin-4: green, LOX: pink). B, Protein-protein interaction between variant fibulin-4 and LOX protein (variant fibulin-4: yellow, LOX: pink).**

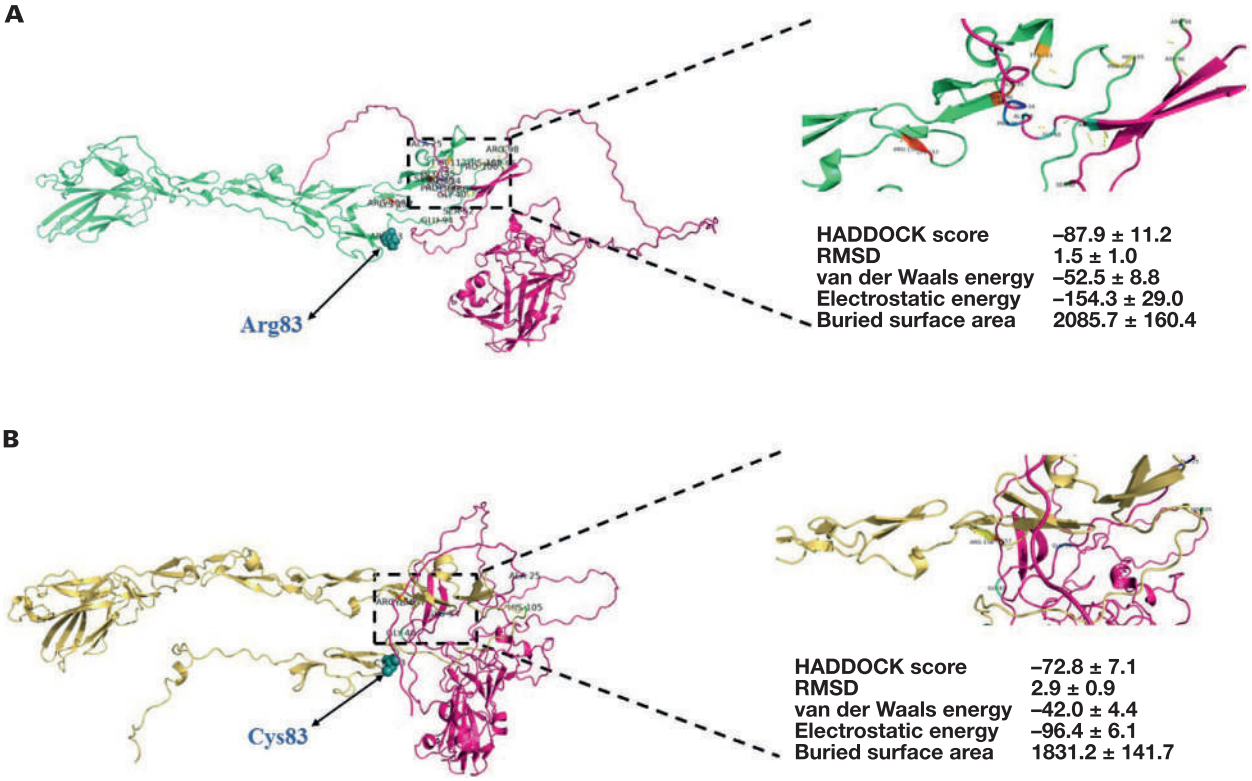
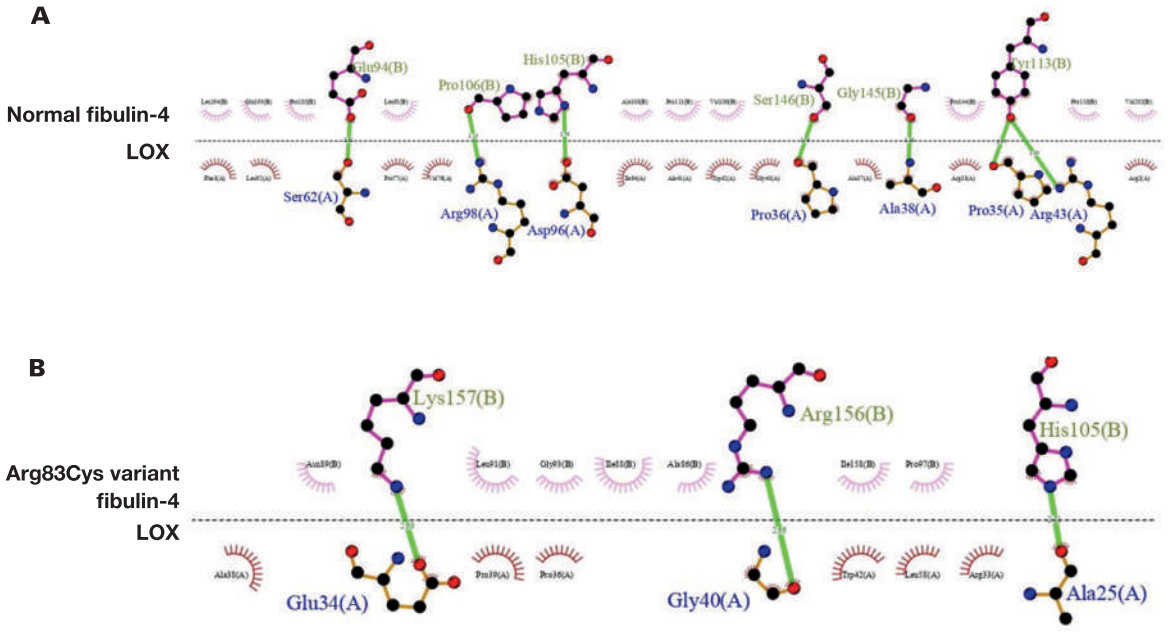


FIGURE 6. **Schematic interaction of the best docking results of normal (A) and variant fibulin-4 (B) with LOX presented by LigPlus+ v.2.2.4. Hydrogen bonding is in green.**



management. Screening of asymptomatic individuals within the pedigree, especially the young, is also recommended. Finally, preventive approaches such as genetic carrier testing for at-risk family members can be implemented.

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The authors acknowledge the kind contribution of the family described herein. This research was funded by the Cardiogenetics Research Center, Rajaie Cardiovascular Medical and Research Center, Tehran, Iran.

Conflict of Interest Disclosure

The authors have nothing to disclose.

Data Availability

The datasets generated and/or analyzed during the current study are available in the ClinVar repository [https://www.ncbi.nlm.nih.gov/clinvar/variation/2430241/].

The accession number of the variant in ClinVar is as follows: NM_016938.5 (EFEMP2):c.247C>T (p.Arg83Cys): VCV002430241.1.

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Urine immunofixation electrophoresis and serum free light chain analyses benefit diagnosis of multiple myeloma in orthopedic patients with normal serum total proteins, creatinine, calcium, and hemoglobin

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Key words: orthopedics, serum free light chains, sFLC κ/λ ratio, immunofixation electrophoresis, multiple myeloma, total protein

Abbreviations: MM, multiple myeloma; TP, total protein; sFLC, serum free light chain; IMWG, International Myeloma Working Group; IFE, immunofixation electrophoresis; CRAB, calcium elevation, renal insufficiency, anemia, bone abnormalities; sLC, serum light chain; GLO, globulin

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ABSTRACT

Background: A substantial number of patients with multiple myeloma (MM) who have bone destruction are initially admitted into the orthopedic service at the hospital. However, routine laboratory testing usually fails to identify these patients, thus delaying optimal therapy. Therefore, there is a clear medical need for early diagnosis of MM in these patients.

Methods: Between 2019 and 2021, 42 patients receiving treatment for orthopedic conditions had normal hemoglobin (Hb), total protein (TP), albumin (ALB), creatinine (CREA), and blood calcium (Ca) levels before their surgical procedure(s) but were subsequently pathologically confirmed to have MM, based on their presenting orthopedic symptoms. During the same period, 52 patients with orthopedic conditions were pathologically excluded from the diagnosis of MM and were recruited into our control group. Serum free light chain (sFLC) testing was performed in 94 consecutive patients in the orthopedic service using Siemens N Latex FLC kits. The levels of Hb, TP, ALB, CREA, and Ca were also measured. All 42 patients with MM were divided into group A (n = 25: κ proliferation) and group B (n = 17: λ proliferation) by the pathology department.

Results: There were no significant differences in levels of Hb, TP, ALB, CREA, and Ca between group A and group B and the control group. However, the sFLC κ/λ ratio of group A and B was also significantly different from that of the control group ($P < .001$). The results of serum immunofixation electrophoresis (IFE) testing demonstrated negative results in 14 cases (58.3%) in group A and 4 cases (25.0%) in group B.

Conclusions: Some patients with orthopedic conditions who do not have typical MM laboratory results, such as those with abnormal Hb, TP, ALB, CREA, and Ca levels before their operation(s), actually have MM. MM should be highly suspected in patients with unexplained bone lesions and with an abnormal sFLC κ/λ ratio. Further tissue or bone marrow biopsy is needed in these patients even if serum and urine IFE results are negative and light chain ratio is normal.

Introduction

Multiple myeloma (MM) is characterized by the clonal proliferation of malignant plasma cells in bone marrow and the presence of paraprotein in serum and urine, all of which are associated with organ dysfunction.[1,2] Elevated levels of serum total protein (TP), creatinine (CREA), and calcium (Ca), and low hemoglobin (Hb) are the typical features of MM in laboratory testing. However, some patients of orthopedic clinics who have MM barely show these typical laboratory features. Serum free light chain (sFLC) evaluation has long been recommended as a part of the International Myeloma Working Group (IMWG) guidelines for MM diagnosis and response assessment.[3] This recommendation was based on the first commercialized sFLC detection assay kit, the Freelite assay (The Binding Site Group), which was developed in 2001.[4] To date, other kits are also available, including the N Latex sFLC κ and λ (Siemens Healthcare Diagnostics), which utilize deviated sFLC κ/λ ratios (reference range, 0.31-1.56) as the indicator of a certain type of abnormal proliferation of monoclonal plasma cells.[5] Although the value of this ratio solely cannot be directly used for making definitive diagnosis of MM, it is strongly associated with a high risk of MM. This ratio can prompt physicians to conduct further relevant examinations, such as blood and urine immunofixation electrophoresis (IFE), bone marrow aspiration, and bone marrow biopsy, to confirm the diagnosis of MM and to select appropriate treatment options.

In this study, we retrospectively analyzed the laboratory values of patients in the orthopedic service who had normal serum TP, CREA, Ca, and Hb levels before their operation but were confirmed to have MM by postoperative pathologic testing, tissue biopsy, or bone marrow biopsy. Our results suggest that the sFLC κ/λ ratio, as determined through sFLC measurement, has great potential in improving the diagnosis of MM in patients in the orthopedic service.

Methods

Patient Selection

This study was approved by the Human Research Ethics Committee of First Affiliated Hospital of Anhui Medical University, Hefei, China, and conducted according to the guidelines of the Declaration of Helsinki. Informed consent was obtained from all subjects and/or their guardian(s). All of the study subjects had bone injury as a clinical symptom and had been admitted into the Departments of Spinal Surgery or Thoracic Surgery from July 2019 through December 2021. The preoperative laboratory values, including serum TP, CREA, Ca, and Hb, were shown to be normal in these patients. MM diagnoses were later made for these patients through pathological examination or bone marrow biopsy. At the same time, patients with thoracic fracture, lumbar fracture, or spinal tumor excluded by pathology were selected as the control group.

Laboratory Analysis

Serum κ and λ FLC concentrations were measured by nephelometry, on a Dade-Behring BN II Analyzer (Siemens Healthcare Diagnostics) based on manufacturer instructions. Serum dilutions were performed according to manufacturer recommendation. We compared the sFLC κ/λ ratios to the published reference range (0.31-1.56).

Serum TP, CREA, and Ca were detected on the cobas 8000 automatic biochemical analyzer, using commercial reagents (Roche Diagnostics). Hb levels were determined using a Sysmex XN-1000 automatic blood cell analyzer, and reagents were commercially provided by Sysmex. IFE was performed using a Hydragel Protein kit and a Hydragel Immunofixation PE kit on a Hydrasys system (Sebia).

Statistical Analysis

Continuous variable data were tested to the validate normal distribution. The results were shown as mean (SD), and comparison between 2 groups of sample data was performed using independent sample *t* testing. Quartiles were used to represent data of skew distribution, and comparisons between 2 groups of sample data were used in Mann-Whitney *U* nonparametric testing. SPSS software, version 26.0 (IBM), and GraphPad Prism 8.0 (GraphPad Software) were used for statistical analysis and mapping. $P < .05$ was considered statistically significant.

Results

Patient Characteristics

In this study, we recruited and analyzed 42 patients of the orthopedic service who had normal serum TP, CREA, Ca, and Hb levels before their operation but had MM pathologically confirmed after their operation or biopsy. These patients were divided into 2 groups: group A (n = 25: κ proliferation) and group B (n = 17: λ proliferation). A total of 52 patients with pathological exclusion of MM after their spinal operation were selected as the control group. The ratios of total light chain and free light chain were normal in these 52 patients.

Comparison of Routine Biochemical and sFLC Analyses

There were no significant differences in Hb, TP, ALB, CREA, and Ca levels between groups A and B and the control group (**TABLE 1** and **FIGURE 1**). However, there were significant differences in the sFLC κ/λ ratio between group A and the control group ($P < .001$) (**TABLE 1** and **FIGURE 2A**). The difference in sFLC κ/λ ratio between group B and the control group was also statistically significant ($P < .001$) (**TABLE 1** and **FIGURES 2B**). There were 5 patients (11.9%) with an sFLC κ/λ ratio within the reference range (0.31-1.56) in the MM group, and all were from group B (**TABLE 2**).

sFLC Response Evaluation for MM Diagnosis

To evaluate the diagnostic values of the sFLC κ/λ ratio for this specific MM population, we created a receiver operating characteristic curve, with sensitivity as the ordinate and 1 – specificity as the abscissa, according to postoperative pathologic testing or bone marrow biopsy results, as the criterion standard. The diagnostic performance of the ratio in patients with MM who had bone injury was area under the curve (AUC) = 1.000 in group A (**FIGURE 2C**) and AUC = 0.9648 in group B (**FIGURE 2D**).

Results of Serum IFE Testing in Groups A and B

Among the 42 patients with MM, a total of 40 had completed identification results via serum IFE (the remaining 2 patients were not tested because the quality and volume of their specimens were insufficient). Serum IFE testing yielded negative results in 14 cases (58.3%) in group A and 4 cases (25.0%) in group B (**TABLE 3**).

Light Chain Only MM in Groups A and B

Among the 42 study-group patients, there were 19 cases of light chain only MM, 12 κ light chain only MM cases, and 7 λ light chain only MM cases. Among the 12 cases of κ light chain only MM in group A, only 4 cases tested positive via serum IFE and the remaining 8 cases tested positive via urine IFE. In all 7 individuals in the group B with λ light chain only MM, serum IFE detection was positive (**TABLE 4**).

Discussion

MM is the second-most-common type of hematological malignant neoplasm in the world.[6,7] SFLC has long been a valuable marker for diagnosing and monitoring light chain MM, amyloid light chain amyloidosis, and monoclonal gammopathy of undetermined significance (MGUS).[8-12] According to the IMWG diagnostic criteria, sFLC assay is recommended for disease surveillance in oligosecretory and micromolecular MM.[13]

Typical patients with MM often have high serum TP, serum CREA elevation, hypercalcemia, and anemia, and patients usually report 1 of the aforementioned symptoms. We discovered that serum TP, CREA, Ca, and Hb values were all within the normal reference range preoperatively in some patients of the orthopedic service; still, those patients were confirmed to have MM via postoperative pathologic testing.

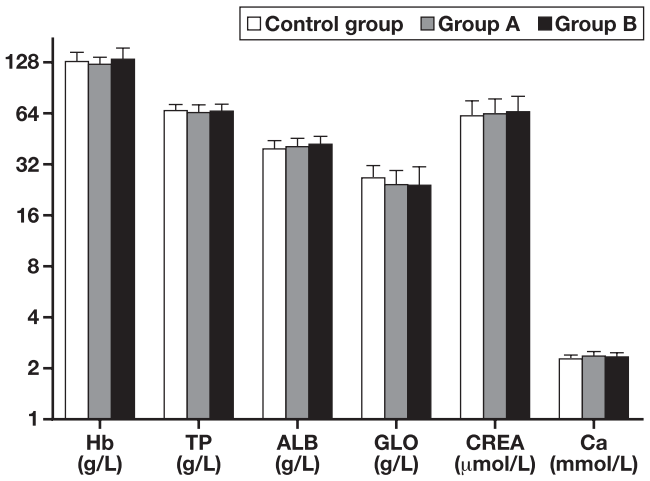
The purpose of this study was to investigate the diagnostic value of sFLC in patients with atypical MM whose clinical manifestations were bone lesions. According to the CRAB (calcium elevation, renal

TABLE 1. Clinical characteristics and analytical data of study participants in the MM and control groups

Characteristic	Reference range	Group A (n = 25: κ proliferation)	Group B (n = 17: λ proliferation)	Control group (n = 52)	*P* value[a]	*P* value[b]
Age, y, mean (SD)		60.6 (9.2)	57.0 (7.6)	67.5 (7.9)		
Age range, y		42-75	48-77	48-78		
Male, No. (%)		15 (60.0%)	10 (58.8%)	32 (61.5%)		
Free κ, mg/L, median (25th-75th percentile)	6.70-22.40	501.0 (218.5-1475.0)	15.50 (9.48-20.40)	21.10 (17.70-27.87)	<.001	.01
Free λ, mg/L, median (25th-75th percentile)	8.30-27.00	19.7 (12.45-34.70)	276.0 (66.50-636.5)	26.90 (21.70-32.07)	.20	<.001
sFLC κ/λ ratio, median (25th-75th percentile)	0.31-1.56	35.00 (9.41-79.04)	0.05 (0.01-0.42)	0.80 (0.68-0.99)	<.001	<.001
Hemoglobin, g/L, mean (SD)	130-175	125.56 (11.96)	135.18 (18.70)	130.08 (16.61)	.23	.29
Range (minimum to maximum)		105-156	105-176			
Total protein, g/L, mean (SD)	65.0-85.0	65.62 (5.79)	66.84 (5.92)	67.21 (4.54)	.20	.79
Range (minimum to maximum)		55.1-77.8	55.1-75.1			
Albumin, g/L, mean (SD)	40.0-55.0	41.10 (4.50)	42.40 (4.29)	40.07 (3.99)	.31	.04
Range (minimum to maximum)		33.2-52.6	34.2-48.8			
Creatinine, μmol/L, mean (SD)	57.0-111.0	64.08 (14.10)	66.12 (14.31)	62.29 (14.09)	.60	.34
Range (minimum to maximum)		45-102	42-91			
Calcium, mmol/L, mean (SD)	2.11-2.52	2.36 (0.14)	2.36 (0.12)	2.30 (0.10)	.06	.09
Range (minimum to maximum)		2.07-2.65	2.17-2.65			

MM, multiple myeloma; sFLC, serum free light chain.
[a]P values between participants (group A and control group) were calculated via the unpaired Student t *test (for the normal distribution) or Mann-Whitney* U *test (for the nonnormal distribution) with continuous variables.*
[b]P values between participants (group B and control group) were calculated via the unpaired Student t *test (for the normal distribution) or Mann-Whitney* U *test (for the nonnormal distribution) with continuous variables.*

FIGURE 1. Hemoglobin (Hb), total protein (TP), albumin (ALB), globulin (GLO), creatinine (CREA), and calcium (Ca) in patients with multiple myeloma (MM) (groups A and B), compared with the control group. There were no significant differences between groups A and B and the control group.



insufficiency, anemia, bone abnormalities) criteria for myeloma target organ damage, the selected 42 patients did not meet the criteria for corrected serum Ca, renal function damage, and anemia, but all met the criteria for bone damage. The sFLC κ/λ ratio of 42 patients with MM who had atypical bone lesions was significantly different from that of the control group without MM after orthopedic surgery ($P < .05$). Therefore, we believe that sFLC testing should be performed in patients with bone destruction even if their serum TP, CREA, Ca, and Hb values are within the normal reference range. This testing can be helpful for the diagnosis of MM and the selection of reasonable treatment options.

We note that the sFLC κ/λ ratio does not provide definitive proof of the presence of monoclonal immunoglobulins (M protein). Any abnormal sFLC κ/λ ratios should be further confirmed via IFE or bone marrow biopsy.[14,15]

International Myeloma Workshop Consensus Panel 3 recognizes serum IFE as the criterion standard to confirm the presence of M protein,[16] which is an important marker of MM in serum. In the present study, serum IFE results were negative in 14 cases (58.3%) in group A and 4 cases (25.0%) in group B. Two of the 18 patients were diagnosed with isolated plasmacytoma, and serum light chain (sLC) and sFLC values were normal, resulting in very low levels of M protein in peripheral blood. In the other 16 cases, although the sFLC detection results were abnormal, the antibodies used in the serum IFE testing were antibodies against total light chain, not antibodies against the specific target of FLC. The concentration of total light chain and FLC in the normal body was approximately 100 times different, so the detection sensitivity limited the detection of trace M protein. Therefore, it is easy to miss MM diagnosis in patients with unexplained bone lesions by only performing serum testing, and it is imperative that urine IFE testing be performed in all such patients.

In this research work, 8 patients with κ light chain only MM tested negative via serum IFE but all of them tested positive via urine IFE. Therefore, in light chain only MM, especially κ light chain only MM, performing serum IFE testing combined with urine IFE testing can reduce the chances for missed diagnosis of light chain only MM. Based on the aforementioned data, it is necessary to perform serum IFE and urine IFE in patients with suspected MM because the sFLC κ/λ ratio is insufficient to include or exclude monoclonal gammopathy.

FIGURE 2. **Serum free light chain (sFLC) κ/λ ratio results of 42 patients of the orthopedic service with normal serum total protein, creatinine, calcium, and hemoglobin levels before their operation but confirmed multiple myeloma (MM) via pathologic testing after operation or biopsy. The 42 patients with MM were divided into group A (n = 25: κ proliferation) and group B (n = 17: λ proliferation) by pathologic testing. **P* < .001. A, Comparison of sFLC κ/λ ratio between MM group A and the control group. B, Comparison of sFLC κ/λ ratio between MM group B and the control group. C, Receiver operating characteristic (ROC) curves of the sFLC κ/λ ratio for patients with MM in group A (area under the curve [AUC] = 1.000; *P* < .001). D, ROC curves of the sFLC κ/λ ratio for patients with MM in group B (AUC = 0.9468; *P* <.001).**

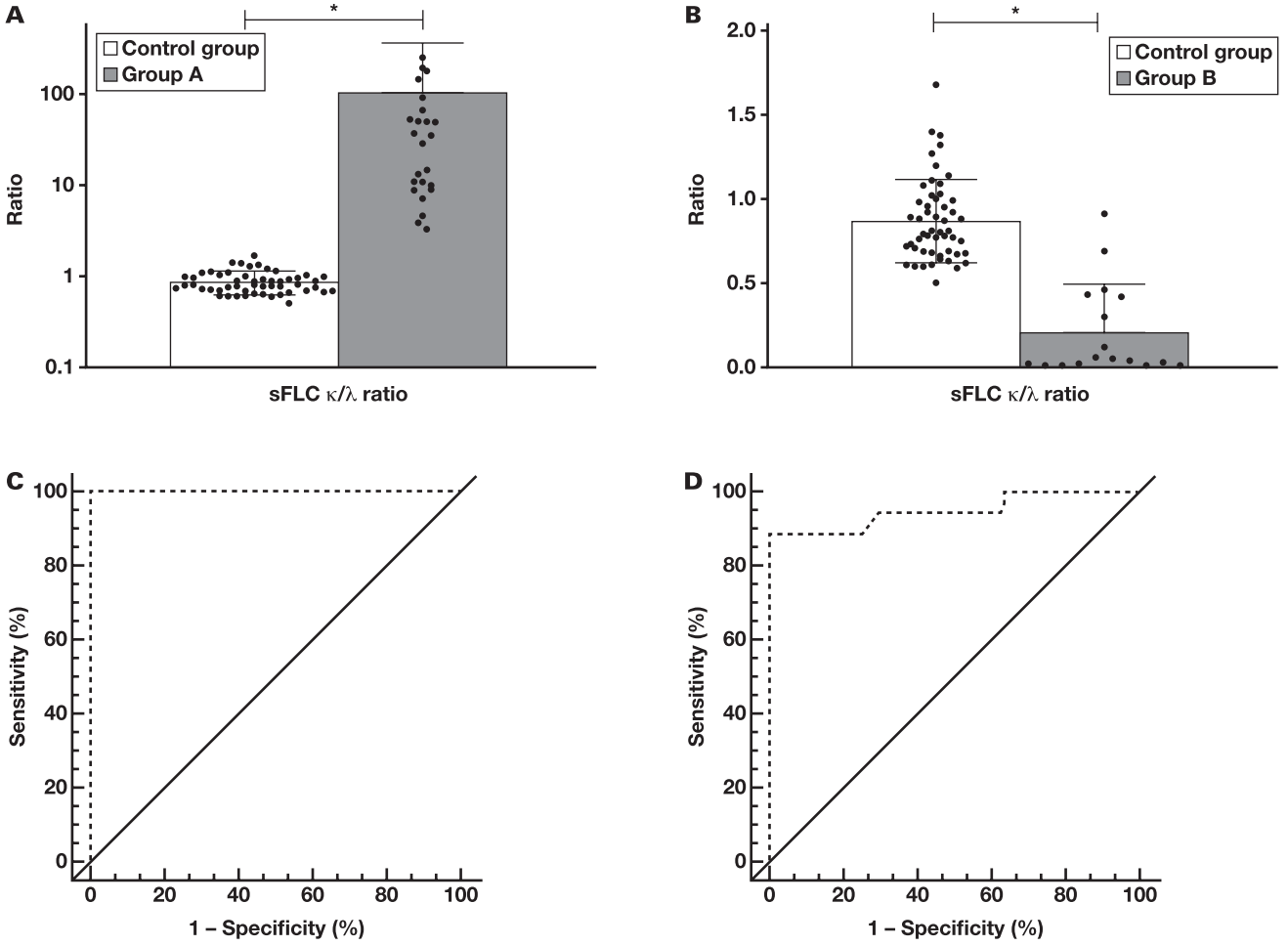


TABLE 2. **Results of sLC, serum IFE, and urine IFE testing in the 5 study patients with normal sFLC κ/λ ratios**

Case No.	Sex	Age, y	Serum κ (g/L)	Serum λ (g/L)	Serum κ/λ ratio	Free κ (mg/L)	Free λ (mg/L)	sFLC /λ ratio	Serum IFE	Urine IFE
17	M	51	1.83	1.22	1.50	14.20	20.60	0.69	Negative	Negative
18	M	77	2.98	2.13	1.40	33.30	36.40	0.91	Negative	Negative
19	F	54	1.66	4.21	0.39	18.40	43.20	0.43	IgAλ	Negative
20	M	54	1.81	6.69	0.27	38.00	89.80	0.42	IgGλ	Negative
21	M	59	1.49	1.76	0.85	19.50	42.10	0.46	IgGλ	Negative
Reference range			1.70-3.70	0.90-2.10	1.35-2.65	6.70-22.40	8.30-27.00	0.31-1.56		

IFE, immunofixation electrophoresis; sFLC, serum free light chain; sLC, serum light chain.

In this study, a total of 5 patients with an sFLC κ/λ ratio within the reference range were found in group B. Although the sFLC κ/λ ratio of 3 of the 5 patients was within the normal reference range, we found that their sLC κ/λ ratio was abnormal and their serum IFE value was positive. Therefore, the combination of sFLC κ/λ ratio, sLC κ/λ ratio, and serum IFE could improve the detection rate of MM. The remaining 2 cases (case 17 and case 18) were confirmed as plasmacytoma via biopsy of the lesion tissue. After positron emission tomography–computed tomography or technetium 99m-methyl diphosphonate whole-body bone imaging, no osteolytic changes were found in parts other than the lesion, and 2 patients were diagnosed with solitary plasmacytoma. These 2 patients were presumed to have nonsecretory M protein MM or it was

TABLE 3. Results of sLC, sFLC, and urine IFE testing in 18 patients with negative serum IFE results

Case No.	Sex	Age, y	Serum κ (g/L)	Serum λ (g/L)	Serum κ/λ ratio	Free κ (mg/L)	Free λ (mg/L)	sFLC κ/λ ratio	Serum IFE	Urine IFE
1	F	54	3.09	0.98	3.12	1520.00	53.20	28.57	Negative	κ light chain only
2	F	62	2.87	1.11	2.59	169.00	18.90	8.94	Negative	κ light chain only
3	F	66	1.83	0.73	2.50	2880.00	19.70	146.19	Negative	κ light chain only
4	F	65	2.32	1.54	1.51	268.00	30.60	8.76	Negative	Negative
5	M	52	1.53	0.47	3.23	441.00	12.60	35.00	Negative	κ light chain only
6	M	56	1.95	1.06	1.84	298.00	27.20	10.96	Negative	Negative
7	M	53	1.50	1.12	1.34	869.00	17.30	50.23	Negative	κ light chain only
8	M	56	2.30	1.56	1.47	153.00	39.60	3.86	Negative	Negative
9	F	46	4.12	1.24	3.32	9300.00	37.10	250.67	Negative	κ light chain only
10	M	67	1.81	0.48	3.75	608.00	12.30	49.43	Negative	Negative
11	M	71	2.21	0.49	4.43	490.00	45.10	10.86	Negative	κ light chain only
12	M	51	2.92	1.51	1.93	336.00	34.00	9.88	Negative	Negative
13	M	60	1.51	0.60	2.51	642.00	12.10	53.06	Negative	κ light chain only
14	F	56	6.72	1.66	4.05	96.90	29.50	3.28	Negative	Negative
15	M	62	0.83	0.69	1.19	21.10	448.00	0.05	Negative	Negative
16	M	68	1.00	0.76	1.30	5.50	621.00	0.01	Negative	Negative
17	M	51	1.83	1.22	1.50	14.20	20.60	0.69	Negative	Negative
18	M	77	2.98	2.13	1.40	33.30	36.40	0.91	Negative	Negative
Reference range			1.70-3.70	0.90-2.10	1.35-2.65	6.70-22.40	8.30-27.00	0.31-1.56		

IFE, immunofixation electrophoresis; sFLC, serum free light chain; sLC, serum light chain.

TABLE 4. Results of sLC, sFLC, and serum IFE and urine IFE testing in 19 patients with light-chain-only MM

Sex	Age, y	Serum κ (g/L)	Serum λ (g/L)	Serum κ/λ ratio	Free κ (mg/L)	Free λ (mg/L)	sFLC κ/λ ratio	Serum IFE	Urine IFE
F	54	3.09	0.98	3.12	1520.00	53.20	28.57	Negative	κ light chain only
F	62	2.87	1.11	2.59	169.00	18.90	8.94	Negative	κ light chain only
F	66	1.83	0.73	2.50	2880.00	19.70	146.19	Negative	κ light chain only
M	49	2.49	0.89	2.80	501.00	13.70	36.57	κ light chain only	κ light chain only
M	52	1.53	0.47	3.23	441.00	12.60	35.00	Negative	κ light chain only
M	53	1.50	1.12	1.34	869.00	17.30	50.23	Negative	κ light chain only
F	46	4.12	1.24	3.32	9300.00	37.10	250.67	Negative	κ light chain only
M	53	2.79	1.31	2.13	904.00	13.60	66.47	κ light chain only	Negative
F	42	1.87	0.59	3.15	1200.00	6.17	194.49	κ light chain only	κ light chain only
M	71	2.21	0.50	4.43	490.00	45.10	10.86	Negative	κ light chain only
M	60	4.31	0.83	5.22	15,500.00	11.80	1313.56	κ light chain only	κ light chain only
M	60	1.51	0.60	2.51	642.00	12.10	53.06	Negative	κ light chain only
M	58	0.88	0.66	1.33	8.67	276.00	0.03	λ light chain only	λ light chain only
M	50	1.96	1.38	1.42	19.70	440.00	0.04	λ light chain only	λ light chain only
M	53	1.35	2.18	0.62	40.60	137.00	0.30	λ light chain only	Negative
F	66	1.53	1.18	1.30	15.50	699.00	0.02	λ light chain only	λ light chain only
M	48	2.15	2.50	0.86	10.60	652.00	0.02	λ light chain only	λ light chain only
F	55	1.75	1.68	1.04	10.30	1350.00	0.01	λ light chain only	Not performed
M	49	1.55	1.03	1.50	15.90	2740.00	0.01	λ light chain only	Negative
Reference range		1.70-3.70	0.90-2.10	1.35-2.65	6.70-22.40	8.30-27.00	0.31-1.56		

IFE, immunofixation electrophoresis; MM, multiple myeloma; sFLC, serum free light chain; sLC, serum light chain.

presumed that their M protein levels were below the detection limit of IFE assay, so this type of patient might need to rely on tissue biopsy to obtain an accurate diagnosis.

Conclusions

The present study data suggest that MM should be highly suspected in patients with unexplained bone lesions with abnormal sFLC κ/λ ratio.

Further tissue or bone marrow biopsy is required in these patients, even if their serum and urine IFE results are negative. In patients with unknown bone lesions, SFLC and urine IFE should be added to serum protein electrophoresis and serum IFE to improve the diagnostic yield of these tests. Because tremendous progress has been made in MM treatments, early and accurate diagnosis of MM is increasingly meaningful in improving prognosis.[17-19]

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Conflict of Interest Disclosure

The authors have nothing to disclose.

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College of American Pathologists Quality Cross Check—Chemistry and Therapeutic Drug Monitoring as a tool for biannual instrument correlations

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Key words: CAP; correlations; regulatory; quality assurance; CZQ; CLIA

Abbreviations: QC, quality control; CZQ, Quality Cross Check—Chemistry and Therapeutic Drug Monitoring; AAB, American Association of Bioanalysts; RCPA, Royal College of Pathologists of Australasia; BMP, basic metabolic profile

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ABSTRACT

Background: Biannual instrument-correlation studies are required for nonwaived assays performed on multiple instruments.

Objective: To determine the feasibility of using College of American Pathologists (CAP) Quality Cross Check—Chemistry and Therapeutic Drug Monitoring (CZQ) to assess instrument correlations among multiple analyzers, analyzer models, and Clinical Laboratory Improvement Amendments (CLIA) licenses for 55 unique analytes.

Methods: Instrument correlation studies were performed on 9 Abbott ARCHITECT instruments (c4000 [n = 4], c8000 [n = 2], and c16000 [n = 3]) over 3 CLIA licenses using CZQ materials. The mean (SD) values, concentration difference, percent bias, and peer data for each individual level of CZQ were determined for each individual analyzer. Acceptable concentration and percentage for each analyte were set using criteria from CAP or other reputable sources such as the American Association of Bioanalysts or the Royal College of Pathologists of Australasia. Peer data were provided by CAP with the CZQ kit.

Results: Correlations using CZQ materials showed that 94.5% of assays studied were within the acceptability criteria by percent bias only and 98.2% were within acceptability criteria by concentration difference.

Conclusions: The use of CZQ provides support to standardized correlation studies among instruments within and across separate CLIA licenses. However, widespread adoption of CZQ may be limited due to concerns regarding matrix effects, analyte ranges, and ease of data analysis.

Introduction

Laboratory tests influence nearly 60% to 70% of all medical decisions.[1] In large institutions, many common and high-volume tests are performed on multiple analyzers or types of point-of-care instrumentation. In the United States, biannual instrument correlation studies for nonwaived tests are required for all analyzers under the same Clinical Laboratory Improvement Amendments (CLIA) license.[2] These correlations help to demonstrate agreement of results among multiple analyzers and methods. Also, although not a regulatory requirement, correlation studies across multiple CLIAs within the same health care system may provide useful information because patients could be tested at affiliated laboratories.

A few approaches can be used to meet the biannual instrument correlation requirement. First, the most common method utilizes residual patient specimens.[3] However, the use of patient specimens can also be fraught with difficulties, especially for institutions with numerous CLIA licenses and different analyzer types (make and model), as well as a large and esoteric test menu. Also, small laboratories may have issues obtaining specimens for analytes that are infrequently ordered, such as several therapeutic drugs, or maintaining stability for analytes such as carbon dioxide (bicarbonate), ammonia, and lactic acid.

Similarly, at a time when clinical laboratories across the United States are experiencing critical medical laboratory professional shortages of 7% to 11%, with some institutions experiencing as much as 25% vacancy,[4] finding ways to decrease the labor burden on laboratory professionals is necessary. In this case, the collection of patient specimens for biannual correlation studies can be time-consuming for laboratory professionals and can take away from critical bench time. Alternatively, the College of American Pathologists (CAP) allows the use of quality control (QC) materials as a substitute for patient specimens. However, this method can be challenging to use because all instruments must be the same

TABLE 1. Correlation analysis for the basic metabolic panel on 3 Abbott ARCHITECT instrument models[a]

Assay	CZQ vial	Peer data	CLIA A														CLIA B		CLIA C	
			c16000-A		c-16000-B		c16000-C		c8000-A		c4000-A		c4000-B		c4000-C		c8000-B		c4000-D	
		Mean (SD)	Difference	% Bias	Difference	% Bias	Difference	% Bias	Difference	% Bias	Difference	% Bias	Difference	% Bias	Difference	% Bias	Difference	% Bias	Difference	% Bias
Bicarbonate	CZQ-01	14.67 (1.60)	2.33	16.0%	−0.67	−4.5%	−0.67	−4.5%	−0.67	−4.5%	−1.67	−11.4%	1.33	9.1%	−0.67	−4.5%	1.33	9.1%	−0.67	−4.5%
	CZQ-02	32.22 (2.40)	2.78	9%	0.78	2.4%	−.22	−0.7%	−1.22	−3.8%	−2.22	−6.9%	0.78	2.4%	−1.22	−3.8%	0.78	2.4%	−0.22	−0.7%
	CZQ-03	21.22 (1.80)	2.78	13%	−0.22	−1.0%	−1.22	−5.8%	−1.22	−5.8%	−1.22	−5.8%	1.78	8.4%	−0.22	−1.0%	−0.22	−1.0%	−0.22	−1.0%
Acceptability: 4 mmol/L or 20% (AAB)																				
Calcium	CZQ-01	12.23 (0.27)	0.15	1.2%	−0.01	−0.1%	−0.21	−1.7%	−0.02	−0.2%	−0.06	−0.5%	−0.18	−1.5%	−0.13	−1.1%	0.18	1.5%	0.27	2.2%
	CZQ-02	6.16 (0.12)	0.09	1.4%	0.01	0.1%	−0.09	−1.5%	0	0%	−0.27	**−4.4%**[b]	0.05	0.8%	−0.01	−0.2%	0.09	1.4%	0.14	2.3%
	CZQ-03	9.79 (0.23)	0.05	0.5%	0.02	0.2%	−0.25	−2.6%	0.02	0.2%	−0.10	−1.0%	−0.12	−1.2%	0.04	0.4%	0.15	1.5%	0.21	2.1%
Acceptability: 0.5 mg/dL or 4% (CAP)																				
Chloride	CZQ-01	88.67 (0.60)	1.33	1.5%	0.33	0.4%	0.33	0.4%	−0.67	−0.8%	0.33	0.4%	0.33	0.4%	−0.67	−0.8%	−0.67	−0.8%	−0.67	−0.8%
	CZQ-02	112.00 (0.90)	0	0%	1.00	0.9%	0	0%	−1.00	−0.9%	0	0	0	0	0	0	0	0	0	0
	CZQ-03	99.00 (0.80)	0	0%	1.00	1.0%	0	0%	−1.00	−1.0%	1.00	1.0%	−1.00	−1.0%	0	0	0	0	0	0
Acceptability: 2 mmol/L or 4% (CAP)																				
Creatinine	CZQ-01	2.11 (0.05)	−0.01	−0.6%	0.04	1.8%	−0.05	−2.5%	0.07	3.2%	0.03	1.3%	−0.08	−3.9%	−0.03	−1.5%	0.02	0.8%	0.03	1.3%
	CZQ-02	0.86 (0.03)	0.01	1.3%	−0.01	−1.0%	−0.02	−2.2%	0.03	3.6%	−0.01	−1.0%	−0.01	−1.0%	−0.01	−1.0%	0.01	1.3%	0	0.1%
	CZQ-03	3.04 (0.07)	0.05	1.5%	0.10	3.2%	−0.07	−2.4%	0.03	0.9%	−0.05	−1.8%	−0.15	−5.0%	0.02	0.5%	0.03	0.9%	0.07	2.2%
Acceptability: 0.1 mg/dL or 7.5% (CAP)																				
Glucose	CZQ-01	298.96 (4.99)	2.14	0.7%	−5.36	−1.8%	−1.36	−0.5%	−3.96	−1.3%	2.04	0.7%	1.74	0.6%	−1.76	−0.6%	10.44	3.5%	−3.96	−1.3%
	CZQ-02	56.94 (0.94)	0.16	0.3%	−1.94	−3.4%	0.86	1.5%	−0.84	−1.5%	0.56	1.0%	0.76	1.3%	−0.94	−1.7%	2.36	4.1%	−0.94	−1.7%
	CZQ-03	183.54 (2.98)	0.76	0.4%	−2.34	−1.3%	0.86	0.5%	−2.54	−1.4%	2.96	1.6%	0.96	0.5%	−3.44	−1.9%	5.36	2.9%	−2.54	−1.4%
Acceptability: 3 mg/dL or 6% (CAP)																				
Potassium	CZQ-01	2.30 (0)	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
	CZQ-02	4.96 (0.05)	0.04	0.9%	−0.06	−1.1%	−0.06	−1.1%	0.04	0.9%	0.04	0.9%	−0.06	−1.1%	−0.06	−1.1%	0.04	0.9%	0.04	0.9%
	CZQ-03	4.01 (0.05)	−0.01	−0.3%	−0.01	−0.3%	−0.01	−0.3%	−0.01	−0.3%	−0.01	−0.3%	−0.01	−0.3%	−0.01	−0.3%	−0.01	−0.3%	0.09	2.2%
Acceptability: 0.2 mmol/L or 5% (CAP)																				
Sodium	CZQ-01	128.89 (0.90)	0.11	0.1%	1.11	0.9%	1.11	0.9%	−0.89	−0.7%	0.11	0.1%	−1.89	−1.5%	0.11	0.1%	−0.89	−0.7%	1.11	0.9%
	CZQ-02	163.44 (1.70)	1.56	1.0%	1.56	1.0%	0.56	0.3%	−0.44	−0.3%	−0.44	−0.3%	−1.44	−0.9%	−0.44	−0.3%	−1.44	−0.9%	0.56	0.3%
	CZQ-03	142.33 (0.80)	1.67	1.2%	1.67	1.2%	0.67	0.5%	−1.33	−0.9%	−0.33	−0.2%	−1.33	−0.9%	−0.33	−0.2%	−0.33	−0.2%	−0.33	−0.2%
Acceptability: 2.5 mmol/L or 2% (CAP)																				
Urea	CZQ-01	43.69 (0.91)	−0.69	−1.6%	0.01	0%	1.01	2.3%	−0.19	−0.4%	0.21	0.5%	−1.19	−2.7%	0.11	0.3%	1.41	3.2%	−0.69	−1.6%
	CZQ-02	20.00 (0.65)	−0.10	−0.5%	−0.30	−1.5%	0.60	3.0%	0.20	1.0%	−0.10	−0.5%	1.00	5.0%	−0.10	−0.5%	−0.20	−1.0%	−1.00	−5.0%
	CZQ-03	30.06 (0.76)	0.14	0.5%	0.04	0.1%	0.34	1.1%	−0.16	−0.5%	−0.76	−2.5%	1.34	4.5%	0.04	0.1%	0.04	0.1%	−1.06	−3.5%
Acceptability: 1.1 mg/dL or 5% (CAP)																				

AAB, American Association of Bioanalysts; CAP, College of American Pathologists; CZQ, Quality Cross Check—Chemistry and Therapeutic Drug Monitoring.

[a]*Abbott ARCHITECT (Abbott Laboratories) instrument models used are the c4000, c8000, and c16000. These models are associated with their Clinical Laboratory Improvement Amendments (CLIA) licenses (CLIA A-C), as well as CAP Cross Check peer data.*

[b]*Bold value is outside the acceptability range.*

make and model, and each assay must be on the same lot of reagent and QC material.[5]

Recently, CAP created Quality Cross Check—Chemistry and Therapeutic Drug Monitoring (CZQ) materials to support external quality assurance schemes. Each kit comes with 3 specimen sets to use in assessing 3 separate analyzers. Kits are mailed twice a year on a set schedule, and custom reports are available with analyte-specific peer data.[6] Also, the commercial material is widely available for many chemistry, immunoassay, and toxicology analytes and could support biannual instrument correlations.

The aim of this study was to determine the feasibility of using CZQ to assess instrument correlations between multiple analyzers, analyzer models, and CLIA licenses. A total of 55 unique clinical chemistry, immunoassay, and toxicology analytes were correlated if performed on at least 2 analyzers (**TABLE 1** and Supplement Table 1). A subset of commonly ordered analytes for which testing was performed on all analyzers is shown, such as calcium and sodium (**TABLE 1**). For a complete list of the 26 common and uncommon therapeutic drugs and 50 other unique clinical chemistry and immunoassay analytes available in the CZQ survey, refer to the CAP survey catalog.[6]

Methods

We ordered 3 CZQ kits and performed analysis using a maximum of 9 Abbott ARCHITECT instruments: the c4000 (n = 4), c8000 (n = 2) and c16000 (n = 3), across CLIA licenses (**FIGURE 1**). After the analysis, the analyte means, concentration differences, and percent bias for each of the 3 individual levels of CZQ were determined for each individual analyzer and analyte in Microsoft Excel, version 16.76 (Microsoft). Concentration difference and percent bias from the mean were calculated using the following equations:

$$Difference = Value - Mean \quad \text{(equation 1)}$$

$$Percent\ Bias = \left[\frac{Difference}{Mean}\right] * 100\%. \quad \text{(equation 2)}$$

Acceptable difference and percent bias limits were set using criteria available by CAP, the American Association of Bioanalysts (AAB), and the Royal College of Pathologists of Australasia (RCPA).

The CZQ data were analyzed by comparing them to the defined acceptability criteria by concentration difference (equation 1) or percent bias (equation 2) if data did not fall within either of the acceptability criteria parameters. Only 1 parameter (ie, difference or percent bias) needed to be within acceptability criteria. For example, as shown in **TABLE 1**, calcium was within the percent bias acceptability criteria (eg, 4%) for all analyzers studied except one (−4.4%, bolded). However, the difference between the CZQ data and the concentration difference was −0.27, which was within the acceptability criteria (**TABLE 1**). Therefore, we determined calcium to be within the acceptability criteria. This approach was applied to all analytes found in **TABLE 1** and Supplemental Table 1.

Peer data reports were provided by CAP on distribution of CZQ kit analyzer results. The data provided in **TABLE 1** serve as examples.

Results

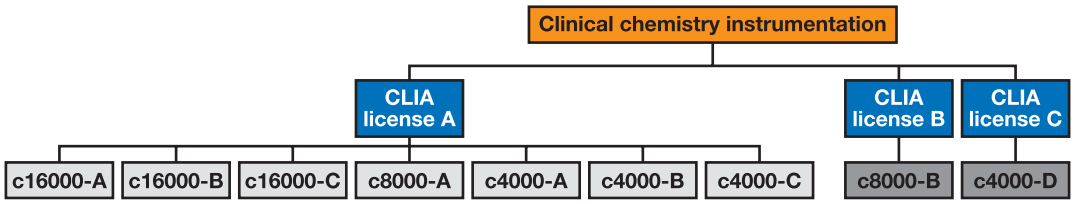
Three separate CLIA licenses, 9 analyzers, and 3 analyzer models were compared in this work of research. Basic metabolic profile (BMP) analysis was performed on all analyzers, and **TABLE 1** lists the specific analytes, with a focus on the 3 different CLIA licenses (labeled CLIA A-C). Acceptability is based on concentration difference and percent bias, to account for analytes with low concentrations. All unique analytes to our test menu in the CZQ (n = 55) were studied; 3 did not meet the percent bias acceptability criteria: total bilirubin, calcium, and methotrexate (Supplemental Table 1). However, when defaulting to concentration difference criteria due to low concentration of the analytes, the correlations were determined to be acceptable for total bilirubin and calcium but not for methotrexate.

Discussion

Correlation studies are an important regulatory technique in the clinical laboratory. They can be useful in determining how well a nonwaived analyte agrees between various analyzers and methods used for patient testing. In this study, correlation results demonstrated that CZQ was able to provide information regarding the precision and accuracy of the 55 unique tests performed among multiple Abbott ARCHITECT instrument models. The results showed that 94.5% of assays were within the acceptability criteria by percent bias only, and 98.2% were within the acceptability criteria by concentration difference. Total bilirubin, calcium, and methotrexate were not within percent bias acceptability range. Variability of certain analytes at lower concentrations may cause large differences by percentage. For this reason, acceptability criteria were included by CAP, AAB, and other organizations as a concentration difference and percent bias.

Methotrexate was the only analyte of the 55 analytes tested that failed to meet concentration difference and percent bias acceptability criteria. As a result, a patient correlation study was performed on all applicable instruments for methotrexate. The results of this analysis showed that methotrexate did meet acceptability criteria with patient specimens (Supplement Table 1). Further studies need to be performed to determine if methotrexate performance within CZQ specimens is comparable to that measurable in patient specimens.

FIGURE 1. Flowchart and correlation analysis of the basic metabolic panel on 3 models of Abbott ARCHITECT instrument (Abbott Laboratories) (c4000, c8000, and c16000) associated with their respective Clinical Laboratory Improvement Amendments (CLIA) licenses (CLIA A-C).



The utilization of CZQ provides a timely and simplified measurement of agreement between instruments, in addition to its ability to compare to measurements in the same category as one another. If implemented, laboratory professionals may decrease the time-consuming search of specific assay values on patient specimens, including those for many therapeutic drugs that, in some laboratories, may not exist. Also, survey kits arrive on a scheduled, biannual timeline, with no need to allot time for laboratory professionals to acquire specimens. Use of the CZQ specimen material also yields decreased run time because multiple assays can be aspirated from the CZQ specimen at the same time, whereas a single patient specimen might be used for 1 assay only. In a time when most clinical laboratories are looking for ways to deal with personnel shortages and to help decrease burnout, CZQ offers laboratories a potential strategy to help alleviate their labor burden.[4]

Limitations of the utilization of CZQ include cost, analyte concentration ranges, and matrix effects. These are key areas to consider with implementation. Although CZQ does provide a potential reduction in required labor, there is a limitation of 3 analyzers that can be assessed per kit. Therefore, the overall costs to perform correlations with CZQ would increase depending on the number of analyzers within a laboratory.

Also, similar to other manufactured and stabilized materials for quality assessment, CZQ materials are limited in their assay coverage ranges (eg, approximately 5%-50% of the linearity range of Abbott assays). However, we note that the material ranges per assay fall within common clinically significant targets and therapeutic ranges. For clinical laboratories serving a smaller patient population, the residual specimens available for correlations may also exhibit a limited linearity range.

Lastly, it has been well established that manufactured specimens resembling serum or plasma can yield different results based on the methodology, instrument, and reagent used (matrix effects).[7] Therefore, it is important to note that CZQ has the potential to be susceptible to matrix effects, and the analysis may be limited to peer group comparisons vs accuracy.[8] However, if the same material is utilized while only changing the analyzer or method used, the ability to measure agreement may still be achievable and could root out a problem to be further assessed (as with methotrexate in our analysis).

Limitation in vial usage across more than 3 instruments raises the question of consistency of analyte value across multiple CZQ vials. However, like QC material, CZQ is produced in large lots, and materials are then aliquoted into vials. These vials are then shipped to participating laboratories under appropriate conditions (eg, temperature) in a similar manner as other laboratory consumables. Therefore, differences between CZQ vials are not expected, although should not be discredited. During our analysis, we did not identify vial variability because this would have been likely recognized in the poor recovery of multiple analytes across the analyzers in the study.

Overall, CZQ testing provides another supportive means to perform correlation studies among multiple instruments within and across separate CLIA licenses. Studies in the future with these types of material should focus on other clinical chemistry instrumentation models or manufacturers, as well as other clinical laboratory analytes. CZQ offers the potential to increase workflow efficiency and reduce labor costs by limiting the time required to locate residual patient specimens. A further commitment from CAP to increase the analyte ranges included and to provide easier data analysis for multiple analyzers within institutional health care systems may make this material better suited for widespread adoption within laboratory medicine.

Supplementary Material

Supplementary material is available at *Laboratory Medicine* online.

Conflict of Interest Disclosure

J.R.W. received an honorarium from Cardinal Health, BioRad, and the Association for Diagnostics and Laboratory Medicine (ADLM) formerly American Association for Clinical Chemistry (AACC), and has received financial support to travel to ADLM, International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), and American Society for Clinical Pathology meetings. J.R.W. served as a consultant with the Cystic Fibrosis Foundation. He currently serves on the document development committee for the first edition of the Clinical & Laboratory Standards Institute (CLSI) PRE06, which will cover external specimen transport evaluations. He is also the chair of the document. He holds 2 provisional patents on SARS-COV-2 antibody testing and serves in leadership roles with the ADLM Serology Task Force COVID-19, Policy and External Affairs Core Committee and the IFCC Task Force on Ethics.

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Implementing point-of-care hemoglobin A1C testing in an obstetrics outpatient clinic

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Key words: point-of-care testing; A1C; pregnancy; diabetes counseling; low resource testing; diabetes

Abbreviations: GDM, gestational diabetes mellitus; T1D, type 1 diabetes; T2D, type 2 diabetes; ADA, American Diabetes Association; POCT, point-of-care testing; ADI, area deprivation index; SES, socioeconomic status; ACOG, American College of Obstetricians and Gynecologists; EMR, electronic medical record; NGSP, National Glycohemoglobin Standardization Program; QC, quality control; DKA, diabetic ketoacidosis; DM, diabetes mellitus

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ABSTRACT

Background: A1C ≥6.0% is associated with increased risk of adverse outcomes in pregnant diabetic patients. A1C testing is recommended by the American Diabetes Association as a secondary measure of glycemic control in pregnant patients.

Objective: To determine the utility of A1C point-of-care testing (POCT) during pregnancy to facilitate rapid counseling and diabetes care, particularly in relatively low-income transient patient populations.

Methods: We performed a single-center, retrospective analysis of patients presenting to an outpatient obstetrics office with routine, in-laboratory A1C testing, before and after the implementation of POCT for A1C (n = 70 and n = 75, respectively). Demographics, results, physician referral to a nutritionist, counseling, and outcomes were retrieved from patient electronic medical records.

Results: In total, 9% and 23% of the in-laboratory and POCT groups, respectively, were referred for nutrition services (P = .02). Of these, 22% of the in-laboratory group and 42% of the POCT group received immediate counseling (P < .01). An inverse correlation was observed between A1C level at study entry and gestational weeks at delivery, with a Pearson r value of −0.39 (−0.58 to −0.16) for the in-laboratory group and −0.38 (−0.57 to −0.14) for the POCT group. No statistically significant difference in pregnancy outcomes was observed.

Conclusion: Implementation of A1C POCT was associated with immediate counseling and management of the health of pregnant patients, but was not associated with improved outcomes, in a low-resource patient population.

Introduction

The incidence of poorly controlled blood glucose during pregnancy has increased in recent years, and the proportion of pregnant patients with gestational diabetes mellitus (GDM) has increased from 6.0% in 2016 to 8.3% in 2021.[1] The proportion of pregnant patients with diagnosed type 1 diabetes (T1D) or type 2 diabetes (T2D) before pregnancy increased by 37% from 2012 through 2016.[2] Sustained hyperglycemia during pregnancy can lead to poor maternal and fetal outcomes, including fetal macrosomia, birth defects, intrauterine fetal demise, preeclampsia, neonatal hypoglycemia, hyperbilirubinemia, and neonatal respiratory distress syndrome.[3]

Further, the risk of obesity, hypertension, and T2D is higher in the offspring of affected pregnant patients relative to those with pregnancies uncomplicated by diabetes.[4,5] Physiologic changes during pregnancy, such as increased red blood cell turnover and the high association of postprandial hyperglycemia with fetal macrosomia, make continuous glucose monitoring the primary method of glucose control in pregnancy. However, the results of studies such as the one by Maresh et al[6] have demonstrated an increased risk of adverse outcomes in patients with diabetes and hemoglobin A1C >6.0%. As a result, the American Diabetes Association (ADA) recommends A1C testing as a secondary measure of glycemic control during pregnancy, with a target A1C of 6.0% without significant hypoglycemia.[7,8]

Point-of-care testing (POCT), or near-patient testing, is commonly performed in inpatient and outpatient settings to provide actionable laboratory results with rapid turnaround time.[9] To this end, POCT has advantages relative to core laboratory testing, particularly in acutely ill patients,[10] patients with diabetes,[11] and perioperatively.[6] However, there are several limitations of POCT, including higher costs relative to in-laboratory testing, its use being associated with increased laboratory error,[12] and at times reduced accuracy.[13] Further, the results of studies such as that by Price[14] have demonstrated that the clinical utility of POCT is dependent on workflow and on a health care provider acting on the POCT result. Therefore, the direct benefits to patient care need

to be assessed, to determine the usefulness of POCT in many cases. Importantly, some studies[15,16] have demonstrated the utility of POCT for underserved populations that often lack access to care and are frequently lost to follow-up.

Studies in nonpregnant subjects have demonstrated improvement in glycemic control and patient satisfaction when using A1C POCT, relative to in-laboratory testing.[17] However, limited data are available in the literature that assess the impact of implementing A1C POCT in pregnant patients. This situation is particularly relevant in patients from economically deprived neighborhoods (ie, high area deprivation index [ADI]), who have reduced access to health care and lower socioeconomic status (SES), who present with a higher incidence of obesity and diabetes, and who have higher rates of GDM but are often lost to follow-up.[16,18]

We note that the results of several studies[19-21] have demonstrated improved diabetes and pregnancy outcomes in patients when care is delivered through a multidisciplinary team that focuses on improved glycemic control of patients with diabetes throughout pregnancy, an approach that is endorsed by the ADA and the American College of Obstetricians and Gynecologists (ACOG).[7] Thus, implementation of A1C POCT represents an opportunity to intervene in higher-risk populations through a multidisciplinary approach that may improve pregnancy outcomes.

The purpose of this study was to assess whether A1C POCT is useful for risk-stratifying pregnant patients for intervention with immediate counseling and referral to a nutritionist in a relatively transient, underserved patient population. Secondary outcomes included the impact of A1C POCT implementation on pregnancy outcomes, including live delivery, term delivery, and requirement for a cesarean section.

Methods

This single-center, retrospective study was approved by the Institutional Review Board of Washington University, St Louis, MO (202108217). A1C results from a single, outpatient obstetrics office were accessed through the laboratory information system (Cerner) in the preimplementation period (in-laboratory group) and in the POCT middleware (Telecore) in the postimplementation period (POCT group). The outpatient obstetrics office of our institution serves a historically underserved neighborhood, with self-paying, uninsured patients coming from neighboring regions with a high ADI.[18] A recent study[18] carried out in the same hospital, serving the same demographic group as in our study, described ADI scores based on United States census block group by considering 17 socioeconomic indicators using the Singh method. ADI percentile was determined using the University of Wisconsin Neighborhood Atlas. ADI, in the sample population, ranged from 30 to 100 by national percentiles, and 60.8% of the patients resided in more-deprived neighborhoods (ADI >85th percentile).

In-laboratory testing was used from January 2017 through January 2019, and POCT testing was used from January 2019 through January 2022. The electronic medical record (EMR; Epic Systems) was queried to procure relevant demographic data and outcomes data using physician-encounter notes. Clinical outcomes assessed included live delivery, weeks of pregnancy, and delivery type. The presence of macrosomia and diagnosis of preeclampsia were assessed but inconsistently documented in the medical record and therefore not included in the analyses. Previous diagnoses of T1D or T2D, or a diagnosis of GDM during pregnancy were recorded. All patient data were collected by a single observer (H.W.). Longitudinal A1C results throughout the pregnancy were captured when available.

Testing in the preimplementation, in-laboratory period was performed in the core laboratory of the institution, via a Roche cobas c502 analyzer (Roche Diagnostics), using the turbidimetric inhibition immunoassay according to manufacturer instructions. This laboratory method is certified by the National Glycohemoglobin Standardization Program (NGSP).

In the postimplementation POCT period, all testing was performed using a Siemens DCA A1C analyzer (Siemens), according to manufacturer instructions, within the clinic of interest. Routine quality control (QC) material was used to assess instrument performance, and weekly patient comparisons were performed between the instruments to compare methods, with a mean bias of +0.2% on the DCA instrument relative to the Roche instrumentation. A1C results were not used to diagnose GDM.

Counseling

Diabetes and nutrition counseling was given to the in-laboratory and POCT groups. For the in-laboratory group, counseling was given based on known previous diagnosis of T1D or T2D. In the POCT group, immediate counseling was also administered if a patient was not known to have diabetes but had A1C of >6%. Due to the turnaround time of results for the in-laboratory group, immediate counseling was not possible for patients who were not known to have A1C of >6%. For counseling, the current dietary history of the patient was obtained, to detect and address patterns of malnutrition. Although nutritional counseling was typically patient-specific, patients were generally counseled to spread out consumption of food into 3 small meals, with preplanned snacks, and guided on the types and amounts of carbohydrates to consume. Referral was also made to the Diabetes Nutrition counseling unit for more specific guidance, which included basic self-management of diabetes with focus on cognitive and management skills. Goal setting was carried out with the patient to denote the motivation of the patient to achieve stronger blood glucose control. Target glucose levels were set, and the patients were trained on glucose meter use and actions to take if experiencing symptoms of hypoglycemia/blood glucose <60 mg/dL, or symptoms of diabetic ketoacidosis (DKA)/blood glucose >250 mg/dL. They were also counseled on the need for diabetes screening 6 to 8 weeks after delivery and yearly thereafter. In addition, patients were educated on proper dosing and intervals of antidiabetic medications.

Statistics

All statistics were calculated using GraphPad Prism 8 (GraphPad Software). Descriptive analysis of data characteristics was carried out to provide the median values and interquartile (IQR) ranges. Categorical data were assessed using contingency tables and χ^2 testing. Student t testing was carried out to compare the mean values of changes in A1C with follow-up tests. Spearman correlation testing was used to assess the relationship between the entry A1C POCT values with weeks of gestation at delivery.

Results

Demographic Information

There were 70 women and 75 women in the in-laboratory and POCT groups, respectively. The median age of the in-laboratory group was 30.0

TABLE 1. Demographic Information for Patients in Our Cohort

Variable	In-laboratory testing (n = 70)	POCT (n = 75)	*P* value
Age, y, median (IQR)	30 (25-33)	30 (26-34)	.80
No. (%) of African American participants	52 (74.3)	62 (82.7)	
A1C value at study entry, %, median (IQR)	6.3 (5.7-7.5)	6.4 (5.7-7.7)	.90
A1C value, No. (%) of population			
<7%	49 (70.0)	47 (62.7)	.30
≥7%	21 (30.0)	28 (37.3)	
No. of gestational weeks at initial presentation, median (IQR)	10.9 (7.4-15.4)	20.3(10.2-31.1)	<.001[a]
Diabetes status No. (%)			
T2D	25 (35.7)	26 (34.6)	.60
T1D	6 (8.6)	11 (14.7)	
Without diabetes	39 (55.7)	38 (50.7)	
Follow-up A1C testing, No. (%)			.40
Yes	23 (32.9)	30 (40.0)	
No	47 (67.1)	45 (60.0)	
A1C improvement at follow-up testing, No. (%)			
Yes	11 (47.8)	13 (43.3)	.80
No	12 (52.2)	17 (56.7)	

IQR, interquartile range; POCT, point-of-care testing; T1D, type 1 diabetes; T2D, type 2 diabetes.
[a]*Performed using unpaired* t *testing. All other statistics performed using* χ^2 *testing.*

years (IQR, 25.0-33.0) and in the POCT group it was 30.0 years (IQR, 26-34; **TABLE 1**). The patients were predominantly of African American race (74% and 83% in the in-laboratory and POCT groups). Median (IQR) A1C at presentation was 6.3% (5.7-7.5) and 6.4% (5.7-7.7) for the in-laboratory and POCT cohorts, respectively, and 30% of patients in both cohorts presented with A1C ≥7%. The presenting obstetrics visit was earlier for the in-laboratory group—10.9 weeks (IQR, 7.4-15.4)—relative to the POCT group—20.3 weeks (IQR, 10.2-31.05). Overall rates of T1D and T2D were comparable between groups. In all, 11% and 24% of women in the in-laboratory and POCT groups, respectively, developed GDM later in the pregnancy. Also, 23% and 30% of women in the in-laboratory and POCT groups, respectively, had follow-up A1C testing performed. Of those, 11% and 13% of the in-laboratory and POCT groups, respectively, demonstrated an improvement in A1C %.

Impact of Implementing POCT of A1C Levels

Immediate counseling was documented for 31.4% of the patients in the in-laboratory group and 56.0% in the POCT group ($P < .01$; **TABLE 2**). Patients previously diagnosed with T1D/T2D were more likely to receive counseling in the POCT group (73%), relative to the in-laboratory-tested group (45.2%; $P = .03$). There was a nonsignificant trend in those without previously diagnosed diabetes, with 40.0% of the POCT group and 20.5% of the in-laboratory group receiving counseling ($P = .08$). More patients in the POCT group (30.7%) received a nutritional services referral, compared with the in-laboratory group (12.9%; $P = .02$). Intrauterine fetal demise was documented in 11.4% of women in the in-laboratory group and 9.3% of the women in the POCT group ($P = .80$). The median (IQR) gestational weeks at delivery were 37.4 (35.6-38.8) and 37.1 (34.9-38.3) for the in-laboratory and POCT groups, respectively. Full-term deliveries (at ≥37 weeks gestation) occurred in 61.4% of the patients who underwent in-laboratory testing and 57.3% of those who had POCT performed ($P = .60$). Of these, 46.2% and 51.3% of these, respectively, were cesarean deliveries.

In patients with serial A1C results, there was no overall trend observed in the in-laboratory group or POCT group (ie, all increasing or decreasing; **FIGURE 1A** and **1B**). There were 4 patients in the POCT group with an A1C value of >10% in the first visit, with a drop to <6.0% in the follow-up visit after 3 months. There were 2 patients in the POCT group for whom the clinicians had concerns of noncompliance with the prescribed regimen, with A1C values increasing by >2.0% at entry to follow-up. The timing of follow-up visits was variable in this transient population. The median times to the first, second, and third follows-ups were 22, 14, and 11 weeks, respectively, in the in-laboratory group, and 12, 8.5, and 8.5 weeks, respectively, in the POCT group (**FIGURES 1C** and **1D**). These intervals were not significantly different between the in-laboratory and POCT groups ($P = .11$). Overall, there was no statistically significant difference between the in-laboratory and POCT groups regarding change in A1C levels over time at each follow-up interval (**FIGURES 1E-1G**).

The delivery date was available to us for 133 of the 145 patients in both groups. A weak inverse correlation was observed between entry A1C and number of gestational weeks at delivery in the POCT and in-laboratory groups (**FIGURE 2A-2B**). The Spearman *r* for both groups were −0.4 (−0.6 to −0.2). The median A1C of patients that delivered before term (<37 weeks; n = 44) was 7.3% (IQR, 6.0-8.8); that value was 5.9% (IQR, 5.4-6.7) for patients who delivered at full term (n = 89; $P < .001$; **FIGURE 2C**).

Discussion

To our knowledge, this is the first study to assess the impact of implementing A1C POCT in pregnant patients presenting in the outpatient setting to the obstetrics department. We observed a significant

TABLE 2. A1C POCT and Pregnancy Outcomes

Variable	In-laboratory testing (n = 70)	POCT (n = 75)	*P* value
No. (%) of pregnant patients peceiving counseling			
Total counseled	22 (31.4)	42 (56.0)	<.01
With T1D/T2D[c]	14 (45.2)	27 (73.0)	.03
Without diabetes[d]	8 (20.5)	15 (40.0)	.08
No. (%) receiving nutritional-services referral	9 (12.9)	23 (30.7)	.02
No. (%) with live delivery	62 (88.6)	68 (90.7)	.78
Median (IQR) gestational weeks at intrauterine fetal demise	37.4 (35.6–38.8)	37.1 (34.9–38.3)	.80[a]
No. (%) of full-term deliveries	43 (61.4)	43 (57.3)	.60
Delivery type, No. (%)[b]			.60
Vaginal	36 (51.4)	36 (48.0)	
Cesarean	31 (44.3)	38 (50.7)	

IQR, interquartile range; POCT, point-of-care testing; T1D, type 1 diabetes; T2D, type 2 diabetes.
[a]Performed using unpaired t *testing. All other statistics performed using Fisher exact testing.*
[b]Delivery type was not accessible for 3 patients.
[c]Percent calculated from the total number of diabetic patients, In-lab = 31 and POCT = 37.
[d]Percent calculated from the total number without diabetes, In-lab = 39 and POCT = 38.

increase in patients counseled for diabetes therapy and immediate nutritionist referral in the offices of physicians in association with POCT implementation. This impact was mostly observed in patients with established diabetes mellitus (DM) diagnoses, particularly T1D. However, we observed no difference in pregnancy outcomes or glycemic control after implementing A1C POCT.

Several studies[7] have demonstrated improved outcomes in women with diabetes who are counseled to improve glycemic control before conception. Diet is pivotal in the treatment of diabetes during pregnancy: following a low glycemic index diet has been associated with improved maternal and fetal outcomes.[22] Therefore, nutritionist referral and immediate consult in high-risk patients is essential to achieving better maternal and fetal outcomes. Although this practice is helpful for patients who have sufficient prenatal care, the guidelines do not address patients with low SES and reduced access to health care. We note that in our study population, median entry A1C levels were higher than the recommended prepregnancy values in >50% of the patients in the in-laboratory and POCT groups and was >10% in ~8% of those patients. Patients with low SES also have a higher likelihood of being lost to follow-up, limiting potential interventions to a single visit.[16]

In this study, we found that adjudication in a high-risk patient population with A1C testing at POCT was associated with an increased likelihood of nutritionist referral and immediate health counseling. POCT has the advantage of a rapid turnaround time relative to in-laboratory testing, facilitating rapid decision-making and influencing health care provider actions. Further, future studies will assess the potential economic impact of implementing POC testing.

Although POC testing is more expensive, including the costs of cartridges and operator training, relative to in-laboratory testing, there is a potential for cost savings if larger studies can demonstrate reduction in cost by improving outcomes (ie, reduced prenatal care after delivery). Together, these results imply a potential benefit of triaging high-risk, pregnant patients with reduced access to health care such that they receive A1C POCT at their initial obstetrics visit.

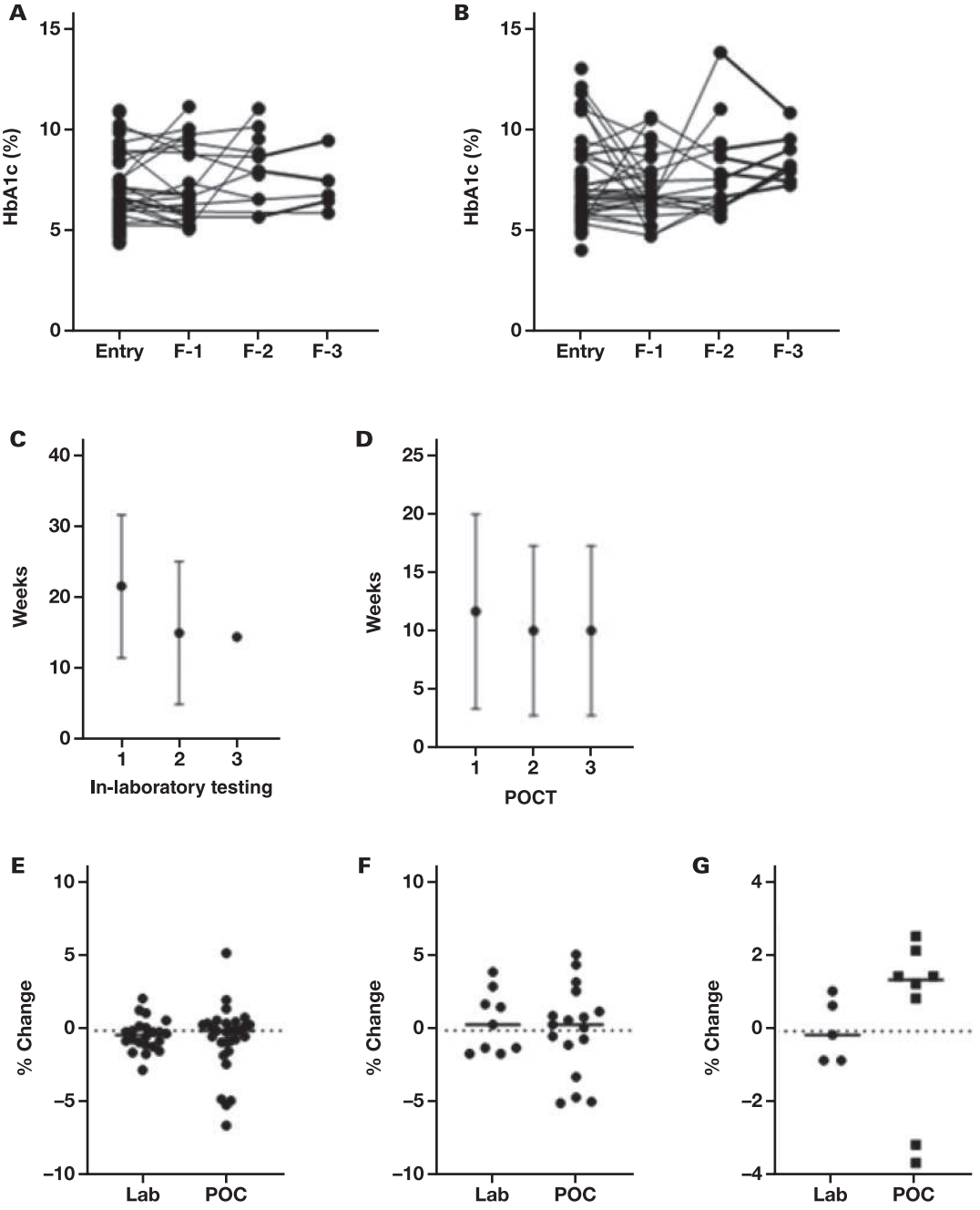
Despite an increase in intervention with counseling on blood-glucose control and immediate referral to a nutritionist, no difference was observed in pregnancy outcomes, including duration of gestation, incidence of live deliveries, or A1C control, after POCT implementation. This result could be due to the limited sample size. Nevertheless, there are limited reports of interventional studies that have used A1C to screen and stratify pregnant patients for treatment. One small randomized trial in pregnant patients with A1C values between 5.7% and 6.4% provided diet counseling, glucose monitoring, and insulin therapy as needed but found no difference in the risk of developing GDM.[23] Another study investigated the effect of preconception lifestyle (diet, healthy weight gain, and exercise) counseling in high-risk groups and found a reduction in labor and postpartum complications.[24] However, studies in patients with pregestational diabetes have demonstrated poorer outcomes in those with A1C >6.5% after 26 weeks gestation.[25]

We observed an inverse correlation between entry A1C value and number of gestational weeks at delivery in the in-laboratory and POCT groups. These results are consistent with those from Maresh et al and imply that A1C testing may provide value for risk stratification in pregnant patients at presentation.[6] Consistent with this finding, patients who delivered before term had higher A1C values at entry in this study, relative to those who delivered at term. Hence, our study findings imply that A1C testing may be a potentially useful screening tool, particularly for prognostication of outcomes in pregnant patients with high risk of T1D and T2D.

However, we urge health care organizations to consider implementation of A1C POCT simultaneously with protocolized, immediate pathways to care. In this study, A1C POCT was initiated with a protocol to provide diabetes counseling and nutritionist referral. Although there was no statistically significant impact on pregnancy-related outcomes for the fetus, previous POCT studies, such as the 2021 study by Nichols,[11] have demonstrated the importance of implementing testing in an optimized workflow to maximize benefits

Other previous studies, such as one by Schnell et al,[17] have demonstrated the clinical benefits of implementing A1C POCT in nonpregnant populations. In our study, improved referral for diabetes education and nutritionist counseling did not improve short-term outcomes. A potential reason that no changes were observed in pregnancy outcomes in this study is that the protocolized interventions may

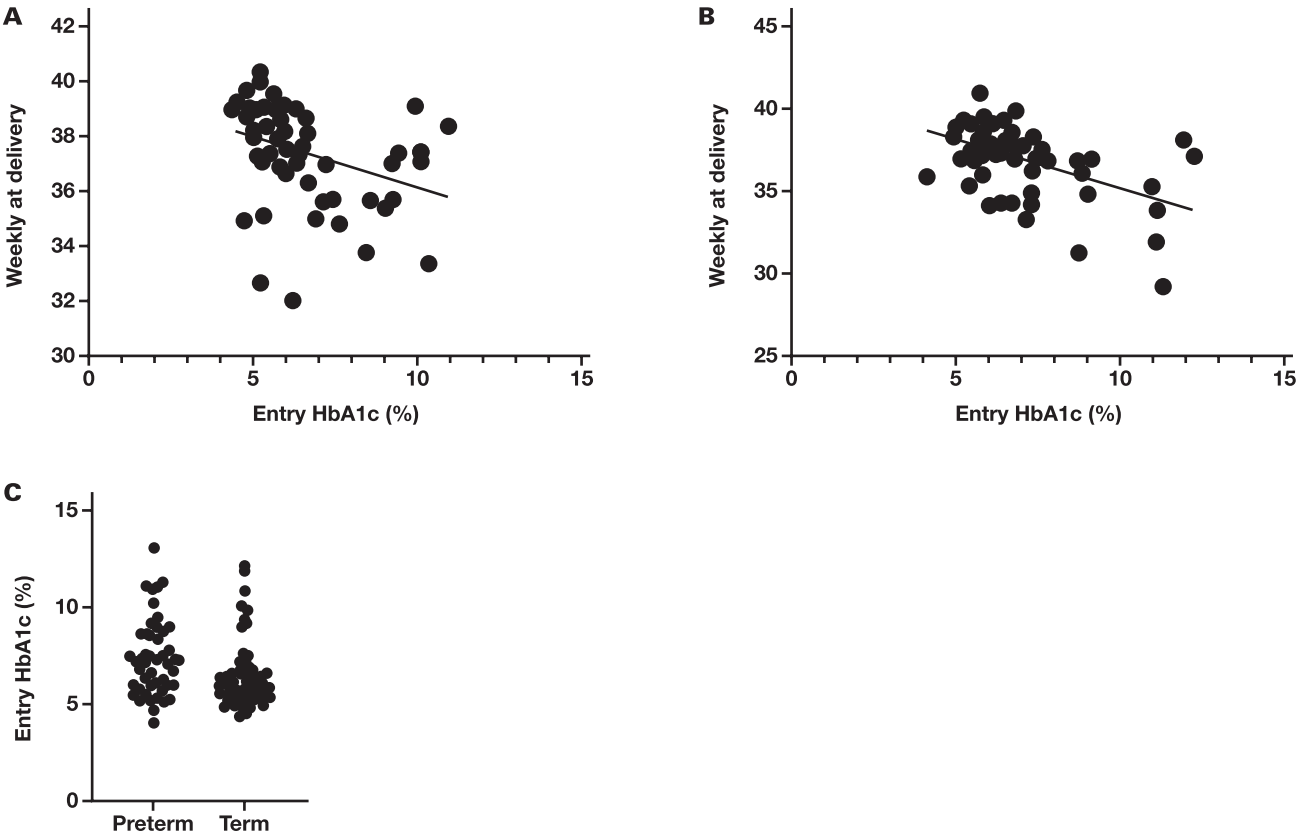
FIGURE 1. **Time course of A1C concentrations. F-1 indicates first follow-up visit; F-2, second follow-up visit; and F-3, third follow-up visit. Change in A1C values over time in patients who received in-laboratory testing (A) and point-of-care testing (POCT) (B). Intervals between follow-up visits in patients who received in-laboratory testing (C) and POCT (D). A1C changes in the in-laboratory and POCT groups from baseline to the first follow-up visit (E), second follow-up visit (F), and third follow-up visit (G).**



be insufficient in the targeted patient population, even after rapid determination of risk using A1C testing.

To this end, social determinants of health, such as patient compliance with health care provider instructions, the affordability of health care, and accessibility to nutritionally well-balanced low-calorie foods, may ultimately influence outcomes more than the interventions used in our study. Further studies are needed to assess more targeted interventions in this patient population. However, the finding that A1C at follow-up did not change significantly in either group implies low patient compliance with interventions. In contrast, it is also unclear how targeted A1C POCT would alter outcomes in an adequately resourced population. We presume that these patients are likely to have better A1C control prenatally, with a lower proportion of patients having a previous diagnosis of diabetes and more likelihood of complying with prescribed lifestyle changes. Further studies are needed to assess the potential impact in this patient population as well. In all, our results imply that larger, and perhaps more controlled, studies in economically stable patient populations are needed to investigate the value of A1C POCT relative to in-laboratory testing.

FIGURE 2. **Relationship between A1C level at study entry and number of gestational weeks at delivery. A, Patient entry A1C level, determined via in-laboratory testing, correlated with number of gestational weeks at delivery (r = –0.4 [–0.6 to –0.2]). B, Patient entry A1C value, determined via POCT, correlated with number of gestational weeks at delivery (r = –0.4 [–0.6 to –0.2]). C, Median entry A1C levels by in patients with term and preterm deliveries (P <.001).**



Our study was limited by sample size and the implementation of POCT in the midst of the COVID-19 pandemic, which may explain the later time at initial obstetrics visit observed in this group. Although the confidence intervals overlapped, implying nonsignificance, the slightly later timing of the POCT group may imply a slight difference in patient populations. This was a single-center, nonrandomized study, which may imply that the results are not generalizable.

Another limitation of this study is that it did not assess the long-term clinical impact of A1C implementation after delivery. Several studies in nonpregnant patients have demonstrated improved, long-term outcomes after implementing A1C POCT.[17] Further studies on the factors influencing the conversion of physician actions, such as counseling and nutrition referral, to lifestyle changes and better pregnancy outcomes would complement the findings in this study. Finally, the retrospective use of the EMR from physician notes has limitations, including the likelihood of missing data and the inability to retrieve laboratory results and physician notes when testing is performed outside our hospital system.

Conclusion

In conclusion, we found that A1C POCT in pregnant patients is associated with rapid decision-making by medical providers, which increased the frequency of counseling, particularly in patients with previously established DM diagnosis and nutrition referral. However, improved pregnancy outcomes could not be demonstrated regarding live delivery, term delivery, and requirement for Cesarean section. Further, larger studies are needed to assess whether A1C POCT improves outcomes in underserved and high-resource patient populations.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Prediction of low-density lipoprotein cholesterol levels using machine learning methods

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Key words: low-density lipoprotein cholesterol, machine learning, prediction model, Friedewald equation, Martin equation, KNHANES

Abbreviations: LDL-C, low-density lipoprotein cholesterol; RMSE, root mean squared error; CVD, AHA/AC, American Heart Association/American College of Cardiology; ESC/EAS, European Society of Cardiology/European Atherosclerosis Society; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; VLDL, very low-density lipoprotein cholesterol; DNN, deep neural networks; KNHANES, Korea National Health and Nutritional Examination Survey; AST, aspartate transaminase; ALT, alanine transaminase; 2step_XGB, 2-step prediction model using XGBoost as a nonlinear model; 2step_RF, 2-step prediction model using random forest as a nonlinear model; MAD, mean absolute difference; NCEP ATP III, National Cholesterol Education Program Adult Treatment Panel III

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ABSTRACT

Objective: Low-density lipoprotein cholesterol (LDL-C) has been commonly calculated by equations, but their performance has not been entirely satisfactory. This study aimed to develop a more accurate LDL-C prediction model using machine learning methods.

Methods: The study involved predicting directly measured LDL-C, using individual characteristics, lipid profiles, and other laboratory results as predictors. The models applied to predict LDL-C values were multiple regression, penalized regression, random forest, and XGBoost. Additionally, a novel 2-step prediction model was developed and introduced. The machine learning methods were evaluated against the Friedewald, Martin, and Sampson equations.

Results: The Friedewald, Martin, and Sampson equations had root mean squared error (RMSE) values of 12.112, 8.084, and 8.492, respectively, whereas the 2-step prediction model showed the highest accuracy, with an RMSE of 7.015. The LDL-C levels were also classified as a categorical variable according to the diagnostic criteria of the dyslipidemia treatment guideline, and concordance rates were calculated between the predictive values obtained from each method and the directly measured ones. The 2-step prediction model had the highest concordance rate (85.1%).

Conclusion: The machine learning method can calculate LDL-C more accurately than existing equations. The proposed 2-step prediction model, in particular, outperformed the other machine learning methods.

Introduction

Globally, cardiovascular disease (CVD) is the leading cause of death,[1,2] and the number of CVD deaths has steadily increased since 1990, owing to aging population growth.[1] Major CVD risk factors have been identified through large-scale cohort studies like the Framingham study and other epidemiologic studies.[3] Low density lipoprotein cholesterol (LDL-C) is the primary target of cholesterol-lowering therapy for the prevention and management of coronary heart disease.[4] Guidelines from both the American Heart Association/American College of Cardiology (AHA/ACC) and the European Society of Cardiology/European Atherosclerosis Society (ESC/EAS) emphasize the importance of focusing on LDL-C for the primary and secondary prevention of atherosclerotic CVD.[5,6]

LDL-C can be measured directly in the laboratory or calculated using an equation. The gold standard for measuring LDL-C is beta quantification using ultracentrifugation, but it is tedious and labor intensive in a typical laboratory setting. Therefore, homogeneous direct assays are often used as an alternative method for measuring LDL-C directly.[7] However, due to the variability in accuracy with manufacturer-specific reagents and the lack of cost-effectiveness in direct homogeneous assays, calculated LDL-C is still widely used in clinical practice.[8] LDL-C is most commonly calculated by using the Friedewald equation, where LDL-C = TC – HDL-C – TG/5 (TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides). The Friedewald equation is derived from a study of 448 patients that found a constant ratio of TGs to very low-density lipoprotein cholesterol (VLDL) of approximately 5:1.[9] However, the fixed factor used to calculate VLDL causes it to underestimate LDL-C in patients with high TG levels. Friedewald recommended that it be used only in patients with TG levels less than 400 mg/dL.[9] In the national health screening program, according to the notification of the Ministry of Health and Welfare in South Korea, LDL-C must be directly measured when the TG level exceeds 400 mg/dL. If not exceeded, LDL-C

should be calculated by the Friedewald equation. Among the 4,819,391 people who received the national health screening program in 2020, only 2% had TG levels greater than 400 mg/dL.[10]

However, the Friedewald equation has some limitations. It is only applicable to sample from fasting subjects,[7] and calculation is inaccurate in secondary hyperlipidemia due to nephrotic syndrome, diabetes mellitus, or liver disease.[11-13] Furthermore, when TG levels are high and LDL-C levels are very low (LDL-C < 70 mg/dL), the Friedewald equation underestimates LDL-C levels.[14,15] This can lead to an undertreatment of high-risk patients.[15,16] The Martin equation, which allows adjustable factor for TG/VLDL depending on TG and non-HDL cholesterol levels, was developed to improve the shortcomings of the Friedewald equation.[17] Both AHA/ACC and ESC/EAS guidelines endorse the use of the Martin equation instead of the Friedewald equation in cases where LDL-C levels are below 70 mg/dL and TG levels fall between 150-400 mg/dL.[18] Wilson et al[8] state that the Martin equation is the preferred method overall, particularly for LDL-C <100 mg/dL and TG 150-400 mg/dL. However, LDL-C calculated with the Martin equation is still inaccurate at high TG levels,[17] so direct measurement of LDL-C is recommended if the TG level is above 400 mg/dL. Recently, Sampson et al[19] developed a new LDL-C equation that uses beta quantification results from patients with a high frequency of hypertriglyceridemia, and they state that their new equation allows for the accurate estimation of LDL-C for individuals with low LDL-C levels and/or TG levels up to 800 mg/dL.

Previous Work

Machine learning methods have been increasingly prominent in clinical research and health care as data science has advanced.[20] Unlike the existing equations that presume a linear relationship between LDL-C and other lipid profiles, machine learning methods can reflect the complex relationship between variables in the model to develop more accurate LDL-C predictive models.[21] As a result, several attempts have been made to develop models to calculate LDL-C more precisely using machine learning methods.[22-27] Singh et al[22] developed a novel LDL-C estimation method with random forest, using a dataset of 17,500 lipid profiles collected from a single medical center. They observed that their method had a higher correlation with directly measured LDL-C than with the Friedewald and Martin equations, including in the setting of elevated TG and very low LDL-C.[22] Kwon et al[23] developed a deep neural networks (DNN) model using medical examination data from a single center and validated their model using an independent dataset. They showed that the DNN model outperformed the Friedewald, Martin, and Sampson equations.[23] Anudeep et al[24] constructed LDL-C prediction models using machine learning methods such as random forest, XGBoost, and support vector machine. Random forest and XGBoost outperformed existing equations in predicting LDL-C.[24] Oh et al[25] used XGBoost and neural networks to construct models for LDL- C prediction. In addition to the lipid profile, they included individual traits and other laboratory test results as predictors. Both XGBoost and neural networks outperformed conventional equations in LDL-C estimation.[25] Tsigalous et al[26] applied various machine learning methods, including multiple regression, support vector machine, XGBoost, and DNN, to estimate LDL-C. They used three datasets consisting of 4,244, 198, and 280 records, respectively.[26] They showed that machine learning methods outperformed the Friedewald and Martin equations, and simple models had comparable performance to deep learning methods.[26]

Accurate LDL-C measurements are essential because inaccurate LDL-C estimation can lead to poor treatment planning and worsen clinical outcomes. In this regard, this study aimed to construct a model that predicts LDL-C more accurately than existing equations, by using machine learning methods and considering individual characteristics (sex, age, body mass index, and waist circumference) and other laboratory values in addition to the lipid profile (TC, TG, and HDL-C) as predictors. We noted that previous studies did not leverage prior knowledge about the strong linear relationship between LDL-C and TC. Therefore, we developed a novel 2-step prediction model, which uses XGBoost or random forest for residuals after applying multiple regression models. This study attempted to compare the prediction performance of our proposed method with that of other methods.

Methods

Data Source and Study Population

The Korea National Health and Nutritional Examination Survey (KNHANES) is a nationwide population-based survey of the health and nutritional status of Koreans. KNHANES comprises a health examination, nutrition survey, and health interview, with the health examination including a blood analysis.[28,29] Direct measurements of LDL-C have been conducted annually for more than a decade. Only individuals with TG levels over 200 mg/dL were given direct LDL-C measurements in all years except 2010, 2011, and 2015. In those years, LDL-C was directly measured in 2400 randomly selected people in 2010-2011 and in all survey subjects in 2015. This study used data from KNHANES 2010, 2011, and 2015. The survey included 8958 participants in 2010, 8518 in 2011, and 7380 in 2015. To meet the criteria of the national health screening program, only subjects aged over 19 were included in the study. Missing values in the study variables were excluded from the analysis. The study sample comprised 1902 participants in 2010, 1928 in 2011, and 4772 in 2015.

All procedures in this study were performed in accordance with the Declaration of Helsinki and all relevant guidelines and regulations. This study was approved by the institutional review board (IRB) of Seoul National University (IRB number: E2211/003-002). The requirement for informed consent was waived because the data used in this study is publicly available.

Study Variables and Measurement

The following factors were taken into consideration when developing machine learning models to predict LDL-C measurements: individual traits like sex, age, body mass index, and waist circumference,[30,31] lipid profiles such as TC, TG, and HDL-C, and indicators of liver function, such as aspartate transaminase (AST) and alanine transaminase (ALT). Patients with chronic renal failure often have higher TG levels due to abnormalities of enzymes involved in lipoprotein metabolism.[32] Therefore, we also considered renal function test results, such as blood urea nitrogen and blood creatinine levels, as predictors. Additionally, we considered diabetes and the use of medication for dyslipidemia as additional predictors, as they may affect the composition of low-density lipoprotein.

Blood samples were collected from the upper arm after at least an 8-hour fast. The lipid profile (TC, TG, HDL-C, and LDL-C), liver function tests (AST, ALT), and renal function tests (blood urea nitrogen, blood creatinine) were measured using the Hitachi Automatic Analyzer 7600. The homogeneous direct assay method, which uses the

Cholestest LDL reagent manufactured by Sekisui, was used to measure LDL-C directly.[28,29]

Statistical Analysis

To present the overall characteristics of the data, categorical variables were presented as numbers and proportions (%) and continuous variables were expressed as median and interquartile range (IQR). The machine learning methods used to predict LDL-C values included multiple regression, ridge, lasso, elastic net, random forest, and XGBoost. In addition, considering that LDL-C has a strong linear relationship with some variables such as TC and exhibits nonlinear relationships with other variables, a 2-step prediction model was newly developed in this study to consider both linear and nonlinear relationships at the same time. The first step involved fitting a linear model, and the combination of variables with the lowest cross-validated root mean squared error (RMSE) was chosen from among all potential combinations. In the second step, nonlinear algorithms, such as random forest and XGBoost, were fitted to the residuals from the fitted linear model. All variables were included when fitting the nonlinear model to the residual. In this study, a 2-step prediction model using XGBoost as a nonlinear model was referred to as 2step_XGB, and a 2-step prediction model using random forest as a nonlinear model was referred to as 2step_RF.

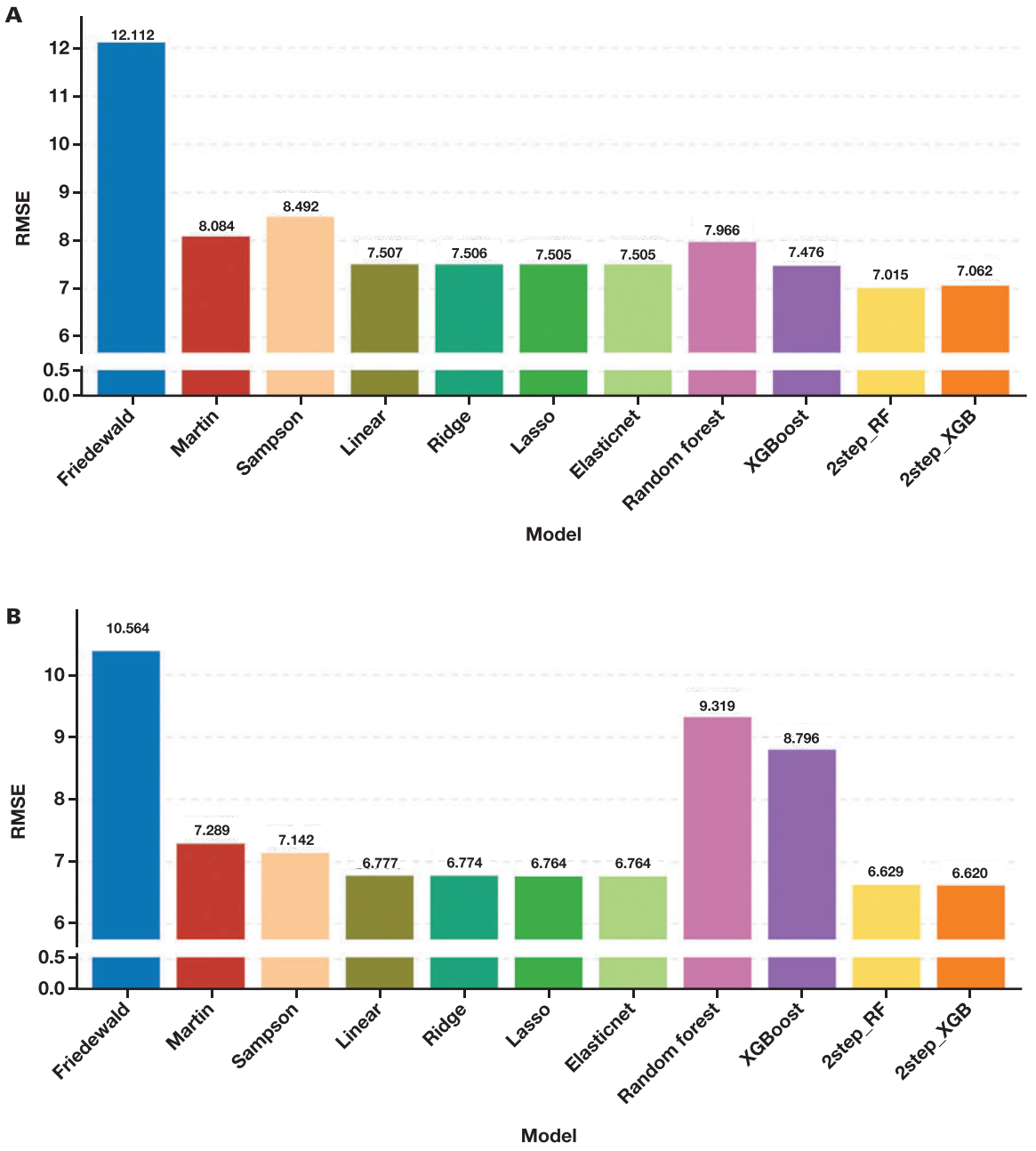
The KNHANES 2015 data were randomly divided into 2 subsets in a 7:3 ratio. The larger subset (70%) was used to create a training set, which was randomly partitioned into 10 equal parts for 10-fold cross-validation to determine the hyperparameters of each method. For ridge and lasso, the tuning parameter is lambda, which controls the magnitude of the penalty term. For elastic net, both alpha (which denotes the relative weight of the ridge and lasso penalties) and lambda are considered as tuning parameters. Random forest has 2 parameters: the number of variables to be randomly selected (mtry) and the number of independent trees to be generated (ntree). XGBoost considers hyperparameters such as the learning rate (eta), the minimum loss reduction required for future splits (gamma), the maximum depth of the tree, and the number of trees (ntree). Supplemental Table 1 displays the range of hyperparameters that were evaluated through cross-validation. The remaining 30% of the KNHANES 2015 data and data from different years were used as separate validation sets to measure the prediction performance of each method. The performance metrics included the RMSE and the correlation coefficient between the directly measured and predicted values. Additionally, the distribution of the difference between the directly measured value and the predicted value was checked according to the TG level to investigate whether the prediction performance varies depending on the TG level. When LDL-C was classified as a categorical variable using the diagnostic criteria for dyslipidemia treatment, the degree of concordance between the predicted and directly measured values was used to evaluate the method. The machine learning methods were compared against the Friedewald equation and

TABLE 1. Baseline characteristics of the study subjects in each dataset[a]

Variable	KNHANES 2015 (n = 4772)	KNHANES 2011 (n = 1928)	KNHANES 2010 (n = 1902)
Age, y	52 (38, 64)	44 (33, 57)	44 (33, 57)
Sex, n (%)			
Male	2086 (43.7)	938 (48.7)	922 (48.5)
Female	2686 (56.3)	990 (51.3)	980 (51.5)
Anthropometric measurements			
BMI, kg/m^2	23.68 (21.50, 25.98)	23.41 (21.27, 25.73)	23.38 (21.27, 25.69)
WC, cm	82.40 (75.60, 89.40)	80.85 (73.50, 88.10)	80.55 (73.70, 87.30)
Lipid profile			
TC, mg/dL	188 (165, 213)	187 (162, 211)	183 (162, 210)
TG, mg/dL	109.00 (74.00, 162.00)	108.00 (71.00, 162.00)	104.50 (70.00, 160.75)
HDL-C, mg/dL	49.35 (41.27, 59.45)	49.35 (41.27, 58.44)	46.93 (40.83, 55.65)
LDL-C, mg/dL	112 (91, 134)	112 (91, 135)	110 (90, 133)
Other laboratory value			
AST, IU/L	21 (17, 25)	20 (16, 24)	20 (17, 24)
ALT, IU/L	17 (13, 24)	17 (12, 25)	17 (13, 25)
BUN, mg/dL	14 (12, 17)	13 (11, 16)	13 (11, 16)
Creatinine, mg/dL	0.81 (0.69, 0.95)	0.82 (0.70, 0.96)	0.80 (0.70, 0.93)
Taking dyslipidemia medication, n (%)			
No	4257 (89.2)	1810 (93.9)	1807 (95.0)
Yes	515 (10.8)	118 (6.1)	95 (5.0)
Diabetes mellitus, n (%)			
No	4218 (88.4)	1766 (91.6)	1746 (91.8)
Yes	554 (11.6)	162 (8.4)	156 (8.2)

ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; BUN, blood urea nitrogen; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol measured by the enzymatic homogeneous assay; TC, total cholesterol; TG, triglyceride; WC, waist circumference.
[a]Data are expressed as medians (interquartile range) for continuous variables and frequencies (percentages) for categorical variables.

FIGURE 1. **Root mean squared error (RMSE) for each method. The RMSE is calculated for each method in the validation set. The RMSE is plotted on the vertical axis, and the bars are color-coded to represent each method. This figure displays the results for 3 validation sets: Korea National Health and Nutritional Examination Survey (KNHANES) 2015 test set (A), KNHANES 2011 test set (B), and KNHANES 2010 test set (C). The 2-step prediction model using random forest as a nonlinear model is denoted as 2step_RF, whereas the 2-step prediction model using XGB as a nonlinear model is denoted as 2step_XGB.**



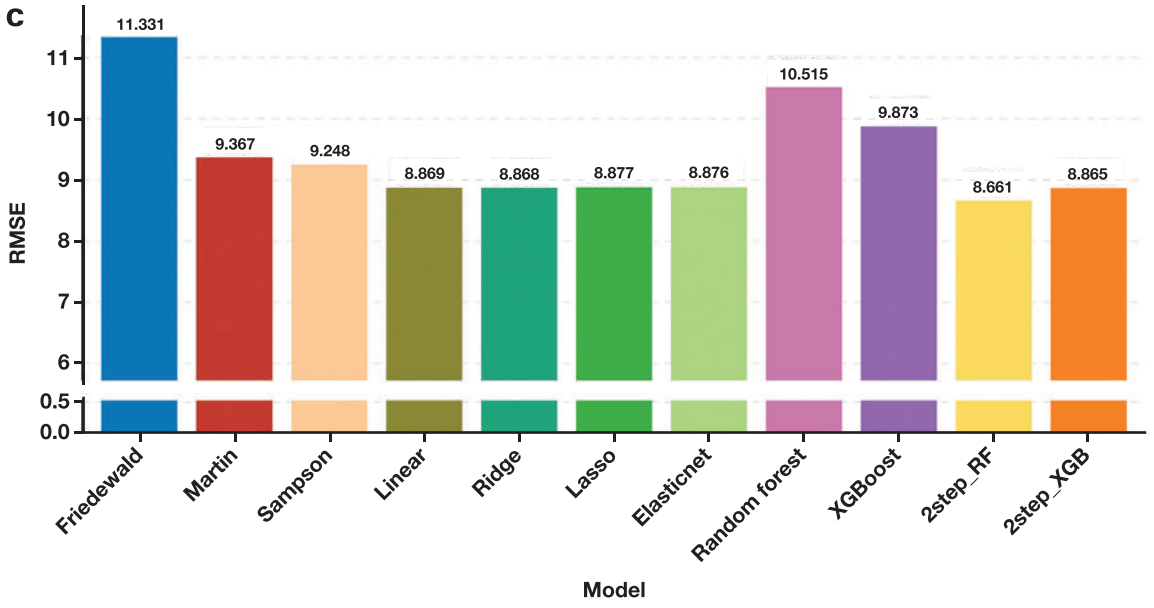
the Martin equation. Statistical analyses were performed using R software (version 4.1.2). Two-sided *P* values of less than .05 were considered statistically significant.

Results

Baseline Characteristics

TABLE 1 presents the baseline characteristics of the study subjects in each dataset. The median age of the subjects in KNHANES 2015 was 52 years (IQR: 38, 64), which was higher than the median age of subjects in the KNHANES 2011 and KNHANES 2010 datasets, whose median age was 44 years (IQR: 33, 57). All datasets had a higher proportion of female subjects than male, and in the KNHANES 2015 dataset, the proportion of male subjects was 43.7%, which was lower than in the other 2 datasets. The median values of lipid profile in the KNHANES 2015 dataset were similar to those in the KNHANES 2011 dataset, but higher than in the KNHANES 2010 dataset. There were no notable differences in other laboratory values between the datasets. In the KNHANES 2010 and 2011 datasets, the proportions of subjects taking dyslipidemia

FIGURE 1. (cont)



medication were 5.0% and 6.1%, respectively, whereas in the KNHANES 2015 dataset, the proportion was 10.8%, which was higher than the other 2 datasets. Additionally, the proportion of subjects with diabetes in the KNHANES 2015 dataset was also higher than in the KNHANES 2010 and KNHANES 2011 datasets.

Root Mean Squared Error

The RMSE was calculated for each method in the validation set (**FIGURE 1**). In the KNHANES 2015 test set, the RMSE of the penalized regression and multiple regression was 7.505 up to 7.507, which was lower than the RMSE of random forest (7.966). The 2-step prediction model yielded the lowest RMSE, with a value of 7.015 for the 2step_RF, outperforming all other methods. In the KNHANES 2011 test set, the RMSE of random forest and XGBoost was 9.319 and 8.796, respectively. In contrast, simple models such as multiple regression and penalized regression had RMSE values ranging from 6.764 to 6.777. The 2step_XGB model showed the lowest RMSE of 6.620 compared with the other machine learning methods. The results from the KNHANES 2010 test set were similar to those from the KNHANES 2011 test set. The RMSE of the penalized regression and multiple regression (8.868-8.877) was lower than the RMSE of random forest (10.515) and XGBoost (9.873). The best-performing method was the 2step_RF model with an RMSE of 8.661, followed by the 2step_XGB model with an RMSE of 8.865. In each validation set, RMSE values for predicted LDL-C by the machine learning methods were consistently lower than those for the Friedewald equation. The RMSEs of predicted LDL-C using the Martin and Sampson equations were higher than those of multiple regression and penalized regression in all 3 validation sets. However, the RMSEs from the 2 equations were lower than those of random forest and XGBoost in the KNHANES 2010 and 2011 test sets.

Correlation Coefficients Between Directly Measured and Predicted LDL-C Values

Correlation coefficient between directly measured and predicted LDL-C was calculated for each method. When the KNHANES 2015 dataset was used as the test set, the Friedewald equation had the lowest correlation coefficient (0.932) compared with machine learning methods. The correlation coefficients for the Martin and Sampson equations were lower than those of penalized regression methods. As shown in **FIGURE 2**, the 2-step prediction model had the highest correlation coefficient (0.974). Similar correlation coefficients were also obtained from the KNHANES 2011 and 2010 test sets. The highest correlation coefficient, 0.980, was obtained from the 2-step prediction models in the KNHANES 2011 test set. Likewise, in the KNHANES 2010 test set, the highest correlation coefficient was obtained from the 2step-RF model (0.964). **FIGURE 2** showed that in the KNHANES 2015 test set, the correlation coefficients of all machine learning methods were equal to or greater than those obtained from the Martin equation (0.967) and the Sampson equation (0.965). In contrast, in the other 2 validation sets, random forest and XGBoost exhibited correlation coefficients that were lower than the Martin and Sampson equations.

Difference Between Directly Measured and Predicted LDL-C Values

Considering that the accuracy of predicted LDL-C values can vary depending on TG levels, we classified TG levels into 5 subgroups: less than 100 mg/dL, 100-149 mg/dL, 150-199 mg/dL, 200-399 mg/dL and over 400 mg/dL. For each subgroup, the mean absolute difference (MAD) between predicted LDL-C and directly measured LDL-C was calculated for each equation and machine learning methods (Supplemental Table 2 and **FIGURE 3**). In the KNHANES 2015 test set, as TG levels increased, MAD values for the machine learning methods and the equations increased, with Friedewald's showing the largest increase. For TG levels below 400 mg/dL, MAD values calculated from the Martin and Sampson equations did not significantly differ from the MAD of machine learning methods. Across all TG levels, the 2step_RF model consistently showed the smallest MAD value. For the KNHANES 2011 and 2015 test sets, the results were similar to each other when TG levels were less than 400 mg/dL. However, when TG was over 400 mg/dL, the prediction performances of random forest and XGBoost were poor in the

FIGURE 2. Correlation between predicted and measured low-density lipoprotein cholesterol (LDL-C) in the Korea National Health and Nutritional Examination Survey (KNHANES) 2015 test set. The x-axis represents directly measured LDL-C values, whereas the y-axis shows the corresponding LDL-C values predicted by different methods. The diagonal line is where y equals x. A, Friedewald equation (r = 0.932; P < .001). B, Martin equation (r = 0.967; P < .001). C, Sampson equation (r = 0.965; P < .001). D, Multiple regression (r = 0.971; P < .001). E, Ridge regression (r = 0.971; P < .001). F, Lasso regression (r = 0.971; P < .001). G, Elastic net regression (r = 0.971; P < .001). H, Random forest (r = 0.967; P < .001). I, XGBoost (r = 0.971; P < .001). J, Two-step prediction model using random forest as a nonlinear model (r = 0.974; P < .001). K, Two-step prediction model using XGBoost as a nonlinear model (r = 0.974; P < .001).

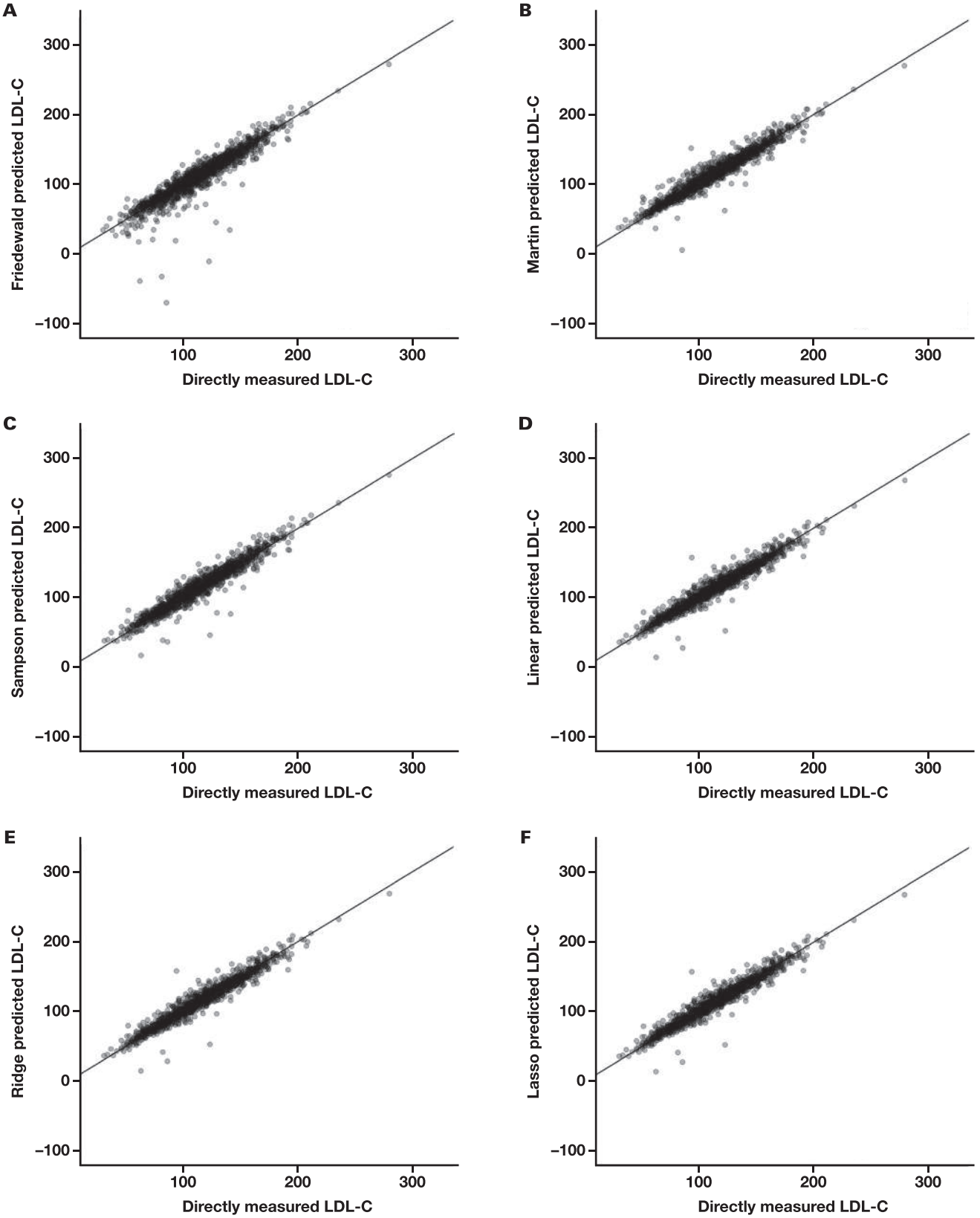
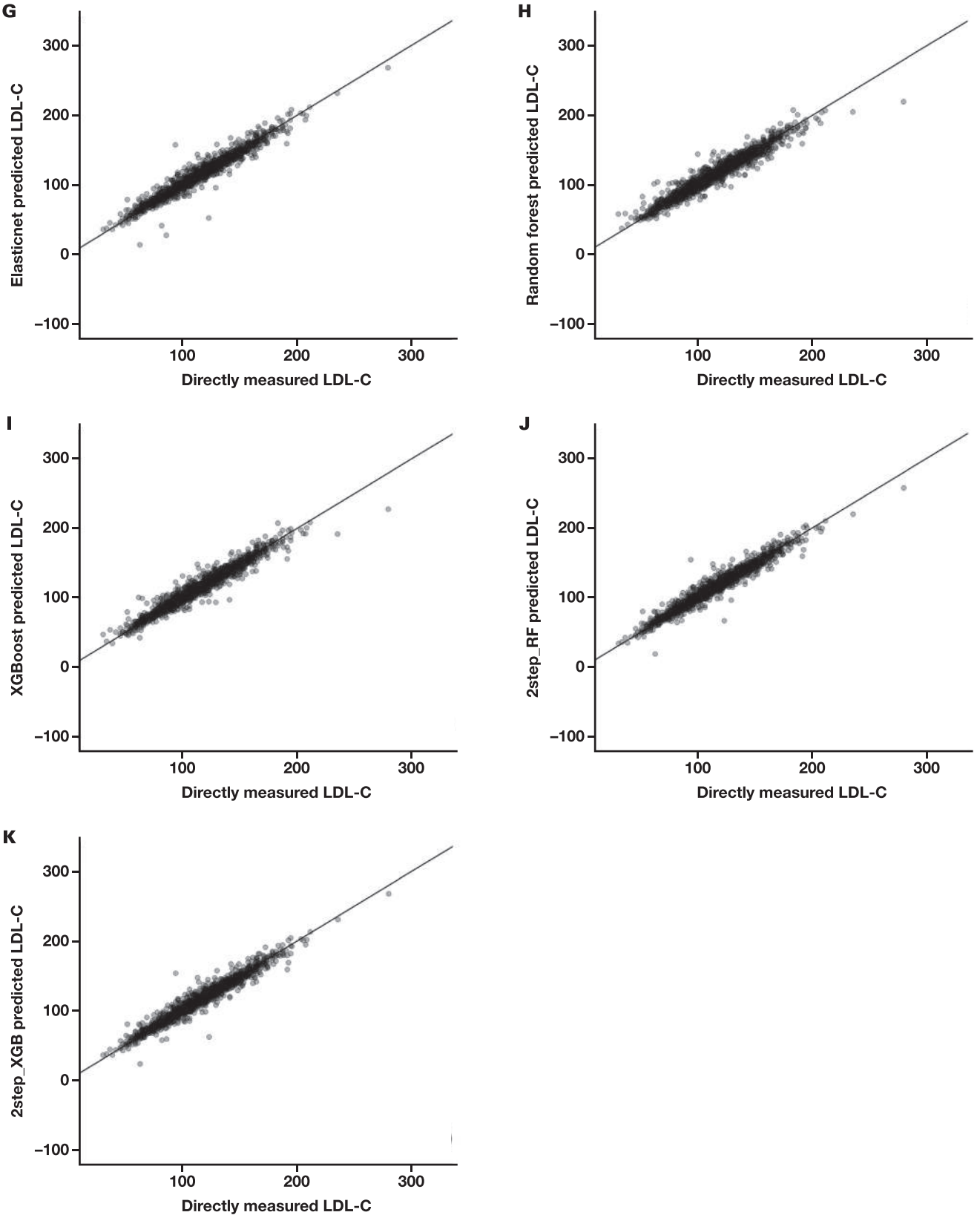


FIGURE 2. (cont)



KNHANES 2011 test set. For TG level less than 100 mg/dL, the 2step_XGB model had the smallest MAD whereas for the remaining range, the 2step_RF model had the smallest MAD. In the KNHANES 2010 test set, the 2step_RF model had the smallest MAD when TG levels were less than 100 mg/dL and between 200 mg/dL and less than 400 mg/dL. For TG levels in the range of 100 to 199 mg/dL, the prediction performances

FIGURE 3. **Mean absolute difference (MAD) for each method. The MAD between predicted low-density lipoprotein cholesterol (LDL-C) and directly measured LDL-C for each method is calculated at different triglyceride (TG) levels. The MAD is plotted on the vertical axis, and the bars are color-coded to represent each method. This figure displays the results for three validation sets: Korea National Health and Nutritional Examination Survey (KNHANES) 2015 test set (A), KNHANES 2011 test set (B), and KNHANES 2010 test set (C). The 2-step prediction model using random forest as a nonlinear model is denoted as 2step_RF, and the 2-step prediction model using XGB as a nonlinear model is denoted as 2step_XGB.**

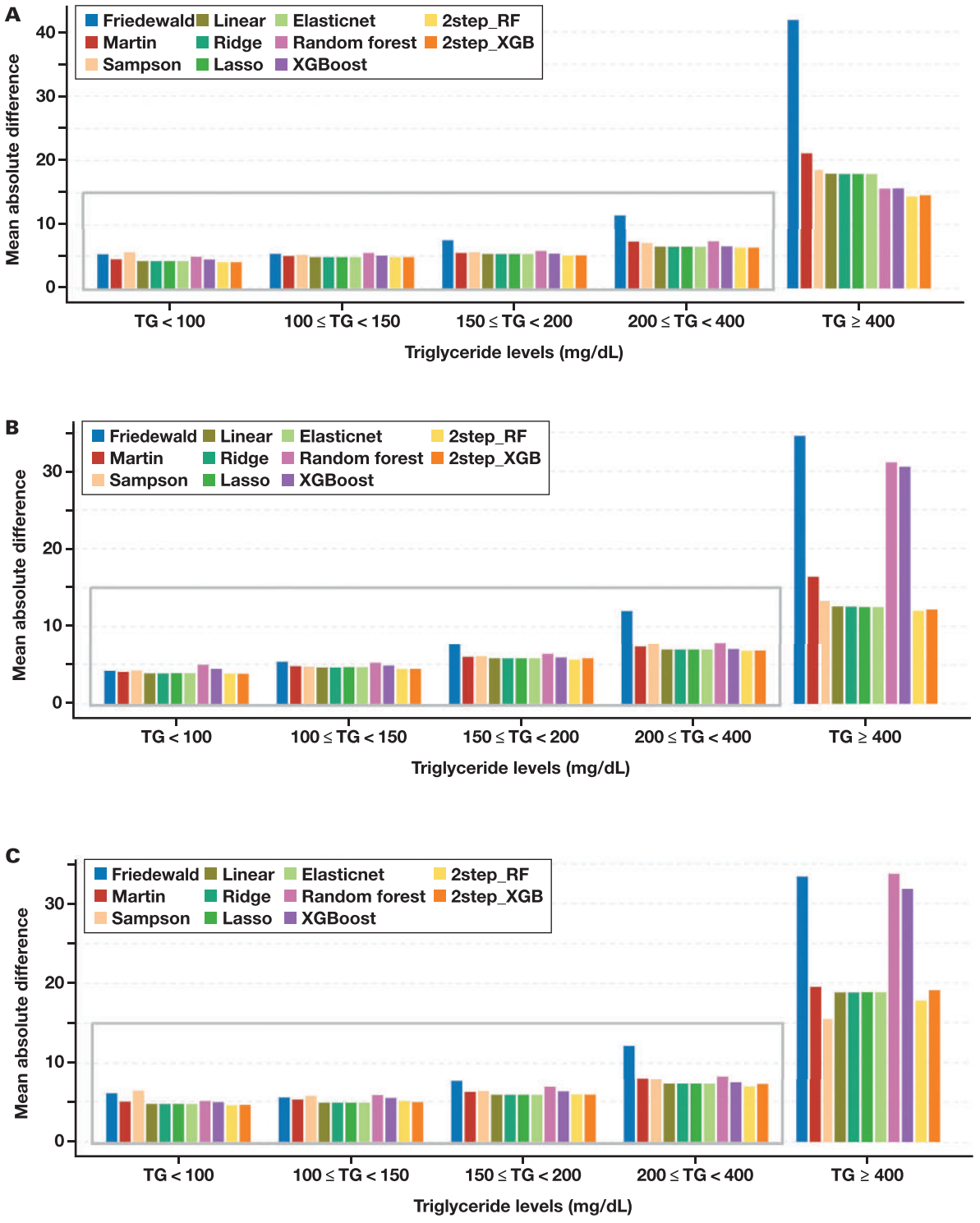
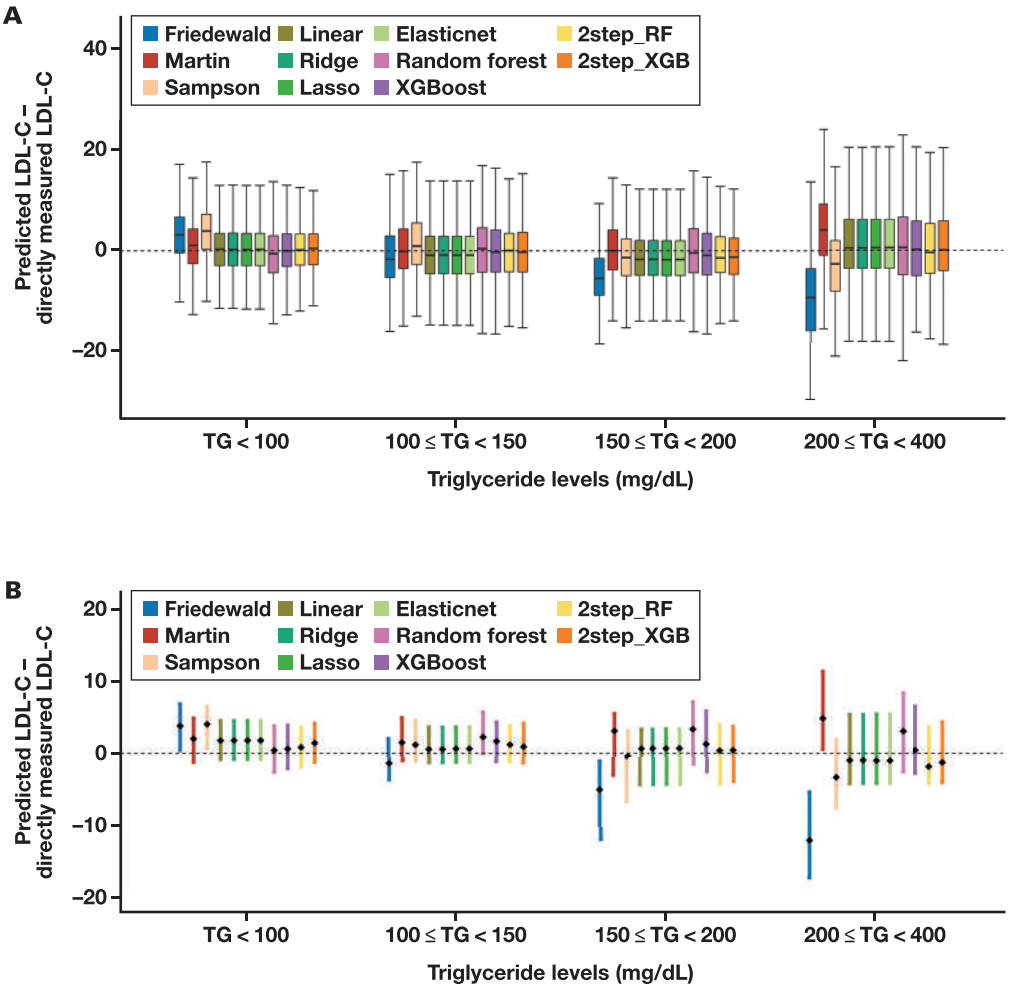


FIGURE 4. Differences between directly measured and predicted LDL-C for each method in the Korea National Health and Nutritional Examination Survey (KNHANES) 2015 test set. Difference between predicted low-density lipoprotein cholesterol (LDL-C) and directly measured LDL-C plotted against TG level, grouped into 4 categories. Each color represents a different equation and machine learning method. A, Box plots show the distribution of differences for each subgroup. B, When directly measured LDL-C is limited to less than 100 mg/dL, the diamond represents median value of each subgroup, and the range of the 25th and 75th percentile values is indicated by each color bar. 2step_RF indicates 2-step prediction model using random forest as a nonlinear model; 2step_XGB indicates 2-step prediction model using XGBoost as a nonlinear model.



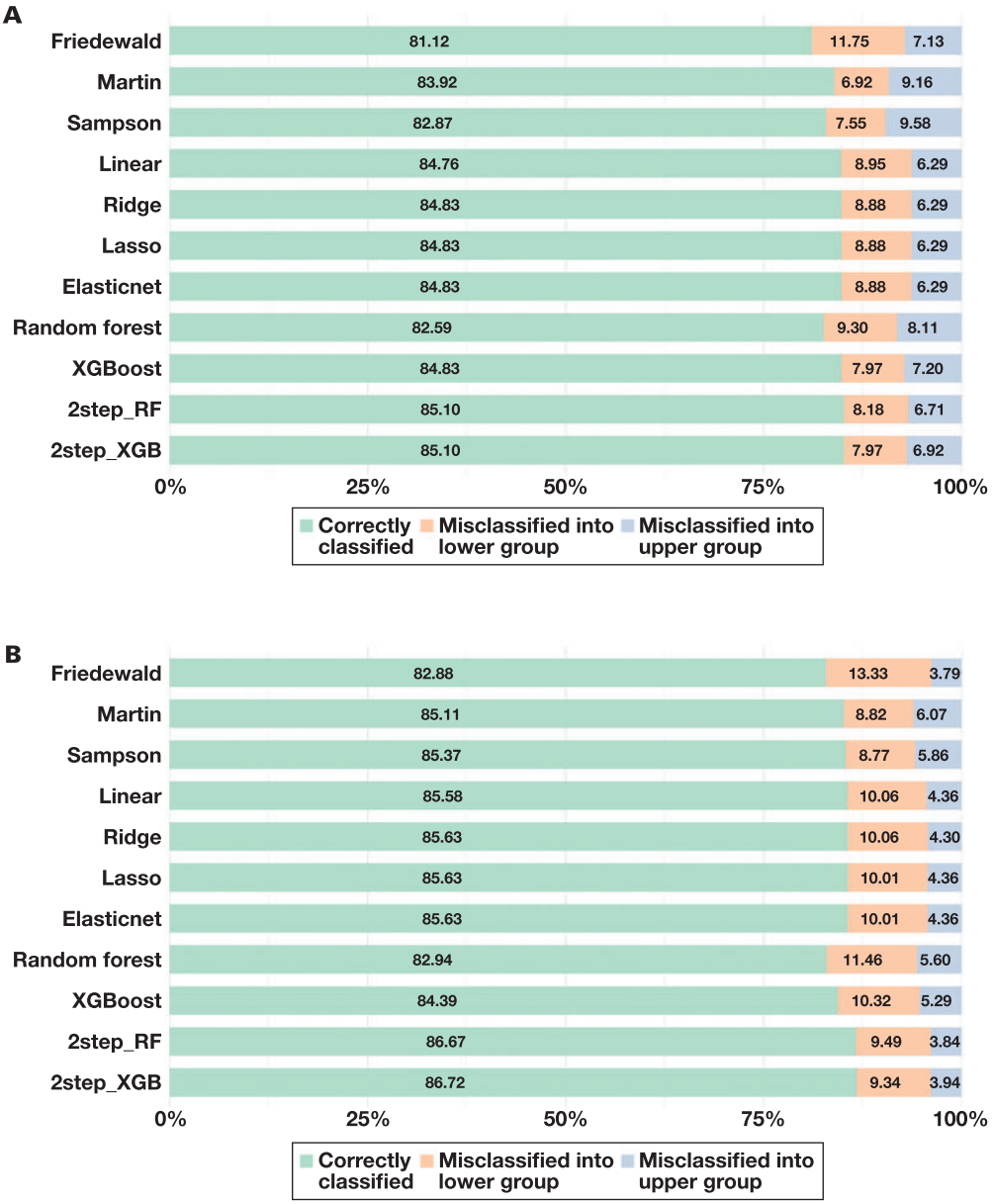
for multiple regression, penalized regression methods, and the 2-step prediction models were almost the same. Unlike the other test datasets, MAD from the Sampson was the smallest when TG exceeded 400 mg/dL.

Comparing Prediction Performances for TG Levels Below 400 mg/dL

To examine the differences between predicted LDL-C and directly measured LDL-C at each TG level, we used boxplots showing the distribution of the differences in **FIGURE 4**. The median of the differences from the Friedewald equation and directly measured LDL-C deviated from zero as TG levels increased. The Martin and Sampson equations yielded median values of differences that were closer to zero or similar to the Friedewald equation (Supplemental Table 3). The Martin equation showed a positive deviation from zero at TG levels of 200-399 mg/dL, and similar results were observed in other validation sets (Supplemental Figures 3 and 4). Similar to the Friedewald equation, the Sampson equation also displayed a tendency to underestimate LDL-C as the TG levels increased (**FIGURE 4**, Supplemental Figures 3 and 4). However, in comparison to the existing equations, the machine learning methods did not show the underestimation pattern based on TG levels in all validation sets.

It is well known that the Friedewald equation tends to underestimate LDL-C levels in individuals with low baseline LDL-C and high TG. Therefore, in cases where LDL-C is very low, we sought to evaluate the prediction accuracy of machine learning methods. Limited to cases where the LDL-C was less than 100 mg/dL, the distribution of differences across TG subgroups is presented in **FIGURE 4** and Supplemental Figures 3 and 4. As TG levels increased, the Friedwald equation showed a greater underestimation of LDL-C compared with directly measured LDL-C, and this pattern was consistent across all validation sets. In contrast, when using the Martin equation, the calculated LDL-C was generally larger than the directly measured value, and the magnitude of the bias also increased as the TG levels increased. For the Sampson equation, as TG levels increased, it exhibited smaller bias in median difference values

FIGURE 5. **Overall concordance rate based on National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) guidelines for each method in each validation set. The bars in the figure represent percentages, with green indicating the proportion of correctly classified subjects, orange indicating misclassification into a lower treatment group, and purple indicating misclassification into an upper treatment group, based on directly measured low-density lipoprotein cholesterol (LDL-C). The figure shows results for the Korea National Health and Nutritional Examination Survey (KNHANES) 2015 test set (A), the KNHANES 2011 test set (B), and the KNHANES 2010 test set (C).**

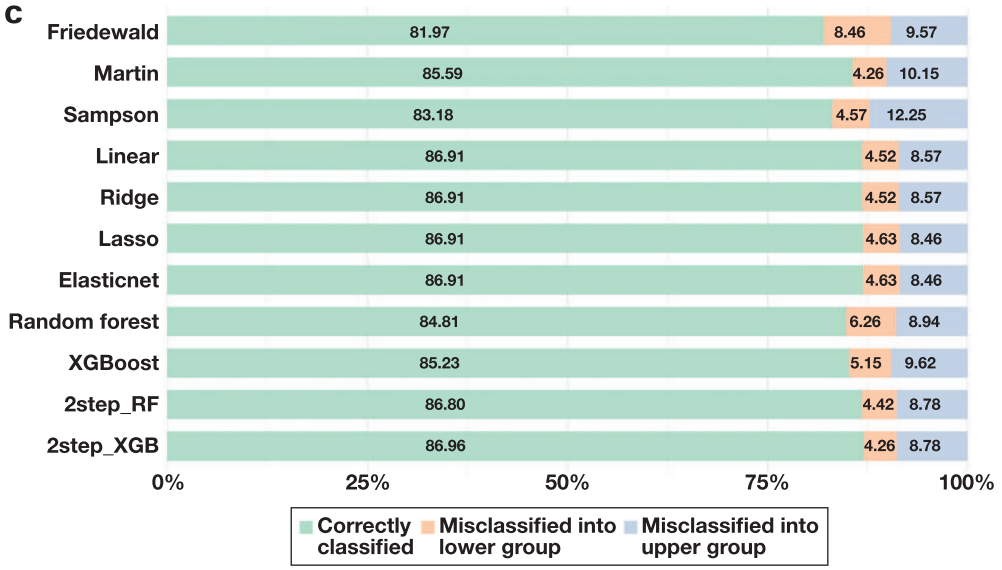


than other equations in all validation sets (Supplemental Table 4). Among the machine learning methods, the multiple regression, the penalized regression methods, 2step_XGB, and 2step_RF provided LDL-C predictions that were similar to the directly measured value, regardless of TG levels.

Comparing Prediction Performances for TG Levels Above 400 mg/dL

Saja et al[33] recently developed the extended Martin equation, which uses strata-specific median ratios of TGs to VLDL for estimating LDL-C levels in patients with TG levels between 400 and 799 mg/dL. The LDL-C prediction performance of the existing equations and the machine learning methods, including the extended Martin equation, was compared for TG levels above 400 mg/dL. Considering that the extended Martin equation was designed for the range of TGs between 400 and 799 mg/dL, we divided the subjects into 2 groups based on a TG level of 800 mg/dL and calculated the MAD value for each method. In the KNHANES 2015 test set, within the TG range of 400 mg/dL to 799 mg/dL, the MAD calculated from the 2step_XGB model was the smallest at 11.372, with the 2step_RF model as the second smallest at 12.016. There was no significant difference from the Sampson equation, with MAD at 12.022. In cases where TG exceeded 800 mg/dL, the 2step_RF model had the

FIGURE 5. (cont)



smallest MAD at 22.093 (Supplemental Figure 5). In the KNHANES 2011 test set, for TG levels between 400 and 799 mg/dL, the 2step_RF had the smallest MAD at 9.532, and the 2step_XGB was the second smallest at 10.044 (Supplemental Figure 6). In the KNHANES 2010 test set, the Sampson equation had the smallest MAD, followed by the MAD of the 2step_RF model (Supplemental Figure S7). As shown in Supplemental Figures 6 and 7, when TG levels exceeded 800 mg/dL, the Sampson showed the smallest MAD in both datasets, followed by the extended Martin equation. However, it's important to note that the number of cases in each validation set with TG levels above 400 mg/dL was relatively small. There were 35 cases in the KNHANES 2015 test set, 44 in the KNHANES 2011 test set, and 40 in the KNHANES 2010 test set. Particularly when TG levels exceeded 800 mg/dL, the cases were very limited, with only 8, 5, and 7 cases, respectively. Therefore, the current result should be carefully interpreted.

Concordance

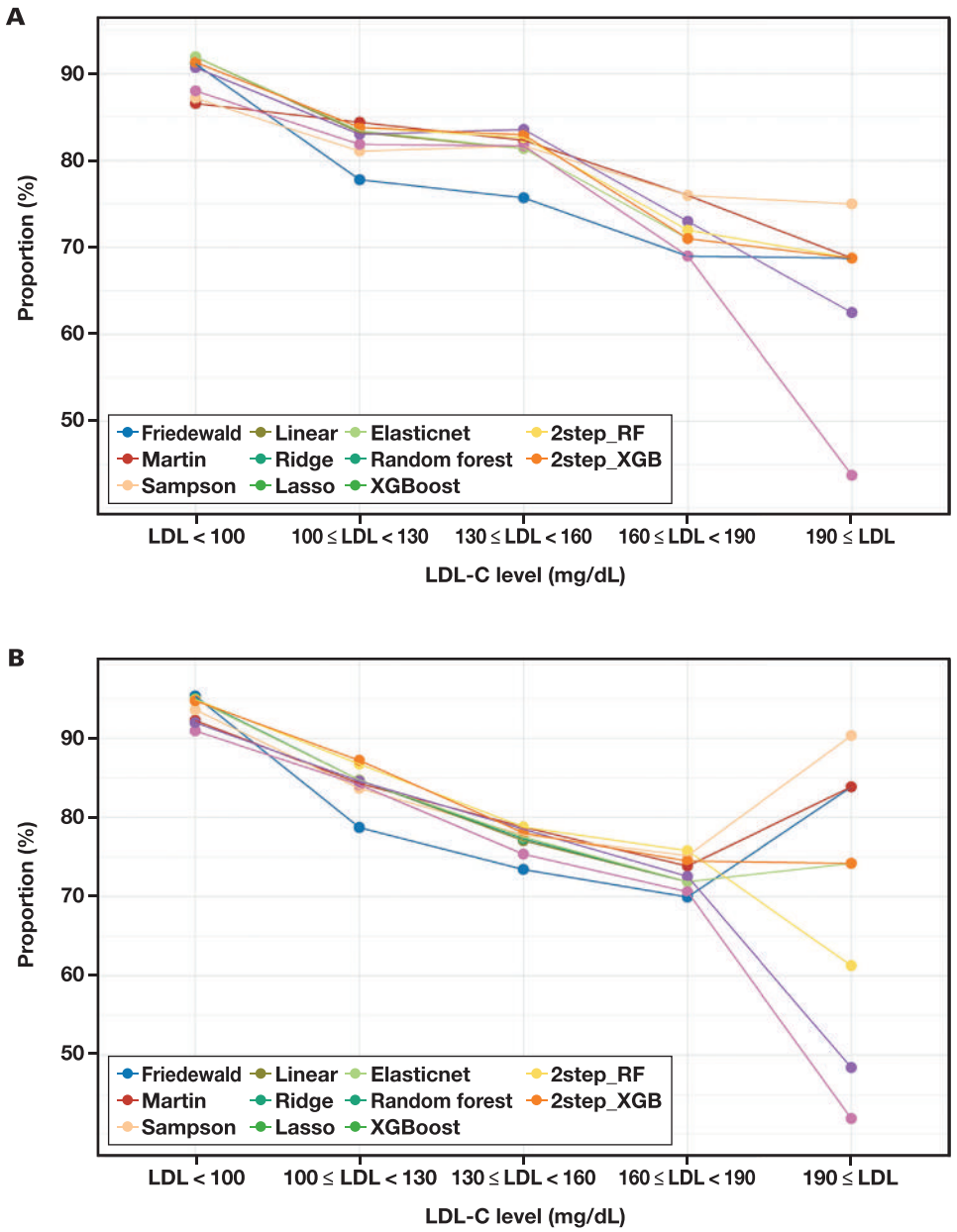
Overall Concordance Rate

The overall concordance rate between directly measured LDL-C values with predicted LDL-C values was evaluated after categorizing LDL-C values into 5 categories based on the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) guidelines. The 5 categories were defined as less than 100 mg/dL, 100-129 mg/dL, 130-159 mg/dL, 160-189 mg/dL, and 190 mg/dL or higher. The concordance rate for each method in each validation set is shown in **FIGURE 5**. The 2-step prediction models achieved the highest concordance rate in the KNHANES 2015 test set. In both the KNHANES 2011 and KNHANES 2010 test sets, the 2step_XGB model exhibited the highest concordance rate, with rates of 86.72% and 86.96%, respectively. In the KNHANES 2011 test set, the 2step_RF model had the second-highest concordance rate, whereas in the KNHANES 2010 test set, the multiple regression and penalized regression methods shared the second-highest concordance rate at 86.91%. The Friedewald equation had the lowest concordance rate in all validation sets, with rates of 81.12%, 82.88%, and 81.97% in the KNHANES 2015, 2011, and 2010 validation sets, respectively. The concordance rate for the Martin equation consistently exceeded that of random forest in every validation set. Multiple regression and penalized regression consistently had higher concordance rates than Martin and Sampson in all validation sets.

Concordance Rate for Subgroups

The concordance rate was compared for each category based on NCEP ATP III guidelines. In the KNHANES 2015 test set, when LDL-C was below 100 mg/dL, multiple regression and penalized regression methods had the highest concordance rate at 91.94%, followed by the 2-step prediction model at 91.32%. In the LDL-C range of 100-129 mg/dL, the Martin equation achieved the highest concordance rate at 84.41%, with the 2-step prediction model as the second highest at 83.82%. In the LDL-C range of 130-159 mg/dL, the top 2 methods with the highest concordance rates were XGBoost and the 2step_XGB model, showing 83.60% and 82.97%, respectively. For LDL-C levels above 160 mg/dL, the Sampson equation showed the highest concordance rate. For the KNHANES 2011 test set, the Friedewald equation had the highest concordance rate for LDL-C below 100 mg/dL. In the LDL-C ranges of 100-129 mg/dL and 160-189 mg/dL, the 2step-XGB and 2step_RF models showed higher concordance rates, respectively. In the range of 130-159 mg/dL, the Martin equation and 2step_RF model exhibited the highest concordance rate at 78.78%. The Sampson equation had the highest concordance rate for LDL-C levels above 190 mg/dL. Similar to the KNHANES 2015 test set, in cases when LDL-C was below 100 mg/dL, the penalized regression method had the highest concordance rate at 88.92%, followed by the 2-step prediction model at 88.64% in the KNHANES 2010 test set. In the ranges of 100-129 mg/dL and 130-159 mg/dL, the highest concordance rates were obtained from the 2step_XGB (86.89%) and the 2step_RF (86.41%). The existing equations were the best for LDL-C levels above 190 mg/dL, where there were only 30 cases (Supplemental Table 7). As shown in **FIGURE 6**, the 2-step prediction model generally showed high concordance rates across all subgroups. A large variation in the concordance rate for the 5th category (LDL-C >190 mg/dL) was observed, and this was due to the small number of individuals belonging to that category.

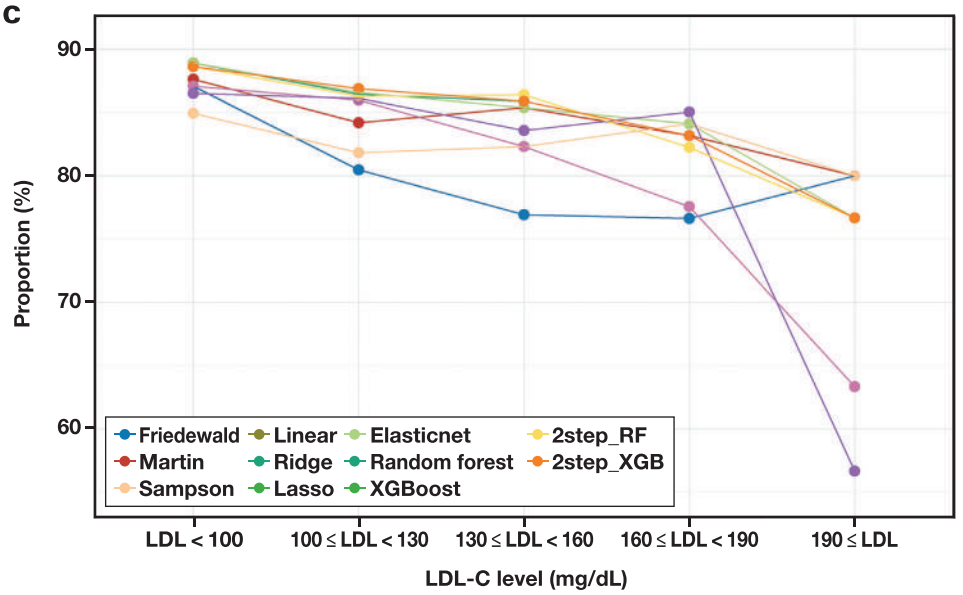
FIGURE 6. **Concordance rate for each low-density lipoprotein cholesterol (LDL-C) subgroup in each validation set. The graph displays the concordance rate for each LDL-C subgroups, with the x-axis representing the subgroups and the y-axis representing the concordance rate. The results from the Korea National Health and Nutritional Examination Survey (KNHANES) 2015, KNHANES 2011, and KNHANES 2010 test sets are shown in A, B, and C, respectively. Each method is identified by its line color. Each color represents a different equation and machine learning method.**



Discussion

All machine learning methods outperformed the Friedewald equation in every performance measure, including RMSE, correlation coefficient, magnitude of bias, and concordance rate. The results of this study are consistent with those of earlier research.[22-26] The Martin equation, developed to overcome the limitations of the Friedewald equation, sometimes showed better performance than XGBoost and random forest but did not outperform the multiple regression or penalized regression methods in every validation set. Singh et al,[22] Anudeep et al,[24] and Oh et al[25] showed that random forest and XGBoost performed generally better than the Martin equation, in contrast to our result. In our study, the Martin equation showed better prediction performance than random forest or XGBoost, especially when TG levels exceeded 400 mg/dL. It appeared that the prediction performance for random forest and XGBoost was influenced by the limited number of cases with TG levels above 400 mg/dL. In general, the 2-step prediction model was shown to be superior to the simple models such as multiple regression or penalized regression, and the simple models were superior to XGBoost and random forest. Similar results were obtained in the study of Tsigalou et al.[26] They constructed an LDL-C prediction model using 4244 samples, similar to the size of the datasets used in this study.[26] The performance of the multiple regression method was better than

FIGURE 6. (cont)



that of the other methods, as shown in our study. However, the performance of the multiple regression was not as good as XGBoost or random forest when a more extensive dataset of 13,391 observations was considered.[24] These results suggest that the model with the best performance may differ depending on the size of the dataset. The performance of the complex model is good when the size of the dataset is large enough, but when it is not, the performance of the simple model can be better. In this study, the Sampson equation exhibited better prediction performance than Friedewald or Martin equation when TG levels were 400 mg/dL or higher. It generally outperformed the extended Martin equation, originally designed for subjects with TG levels between 400 mg/dL to 799 mg/dL. In all validation sets, the prediction performance of the proposed 2-step prediction model was generally better than that of the Sampson equation in the TG level between 400 mg/dL to 799 mg/dL.

Recent research has demonstrated that the retention of LDL-C within the artery wall is the primary beginning event in atherogenesis. Furthermore, these clinical trials have clearly shown that the lower the LDL-C values reached, the lower the risk of future cardiovascular events, with no lower limit for LDL-C values.[6] Although it is ideal to adopt beta quantification as the reference method to quantify LDL-C, beta quantification is typically challenging to apply because it requires much time and effort.[7] Although the homogeneous direct assay is an alternative to beta quantification, there is still a cost burden, unlike with the Friedewald equation. According to the cost of the national health screening program, 1 LDL-C measurement costs about 5 dollars. If LDL-C levels were directly measured, even if TG levels were below 400 mg/dL among people receiving the 2020 national health screening program, it would cost approximately 24 million dollars. This study showed that using the proposed 2-step prediction model can yield LDL-C values that are more accurate than those given by the Friedewald equation without losing cost-effectiveness.

Study Limitations

This study has some limitations. First, in addition to the relatively small size of the dataset used in this study, blood samples are typically obtained from healthy individuals by the KNHANES examination guidelines. Therefore, there is a lack of information regarding the case for aberrant lipid profiles in model development. For instance, there are few individuals with TG levels larger than 400 mg/dL in our dataset. The proportion of individuals with diabetes and the use of medication for dyslipidemia was small. Therefore, further research will be necessary to compare the performance of the 2-step prediction model with that of other machine learning methods using large datasets. Second, KNHANES adopts a homogeneous direct assay method to detect LDL-C rather than the beta quantification corresponding to the gold standard method.[27,28] It is not yet clear whether the proposed 2-step prediction model will also show the best prediction performance when using beta quantification. Therefore, further research will be necessary to compare the performance of the 2-step prediction model with that of other machine learning methods using the LDL-C measured from the beta quantification. Third, the prediction performance for LDL-C can be improved by using detailed electronic health records. Therefore, further research is needed to determine how much the additional information will enhance the prediction performance of the machine learning methods.

Conclusion

The prediction performance of the machine learning methods outperformed the Friedewald equation in this study. When LDL-C is calculated using the existing equations, the bias increases in the groups with higher or lower TG levels, whereas the machine learning method showed a small bias regardless of TG levels. In general, the machine learning method can calculate LDL-C with more accuracy than the existing equations. The proposed 2-step prediction model, in particular, outperformed the other machine-learning methods. Therefore, the 2-step prediction model can be recommended to predict LDL-C levels.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Autoantibodies in laryngeal cancer: detection and role as a biomarker

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Key words: tumor immunology; head and neck cancer; laryngeal cancer; autoantibodies; serological biomarker; ELISA; diagnostics

Abbreviations: AAb, autoantibodies; LC, laryngeal cancer; PPV, positive predictive value; NPV, negative predictive value; LR-, negative likelihood ratio; LR+, positive likelihood ratio; TAA, tumor-associated antigens; PSA, prostate-specific antigen; ELISA, enzyme-linked immunosorbent assay; ATCC, American Type Culture Collection; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulphate; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; PBST, PBS with Tween 20; OD, optical density; ROC, receiver operating characteristic; ANOVA, analysis of variance; AUC, area under the curve

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ABSTRACT

Objective: Diagnostic role of autoantibodies (AAb) as serological biomarkers has not been specifically investigated in laryngeal cancer (LC) previously. The study investigates the presence of anti-LC AAbs and their potential as a biomarker for early diagnosis of LC, to improve patient outcome.

Method: Anti-LC AAb levels were investigated in LC patients (n = 30) and healthy individuals (n = 30) by indirect enzyme-linked immunosorbent assay (ELISA). Patient AAb levels were analyzed with various clinical factors, primarily tumor stage.

Results: AAb levels were significantly higher in LC patients than in the control group (P = .019). The diagnostic performance of AAb-level testing for LC detection presented a sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of 70% each. The positive likelihood (LR+) and negative likelihood (LR–) ratios were 2.33 and 0.43, respectively. AAb levels were independent of cancer stage (P = .708), duration since first appearance of symptoms (P = .228), duration of medical attention (P = .231), and degree of risk-factor exposure (P = .478).

Conclusion: Significant level of AAbs could be detected among LC patients with good diagnostic performance, irrespective of stage. Thus, anti-LC AAbs reflect potential to be utilized as predictive biomarkers in early diagnostics of LC.

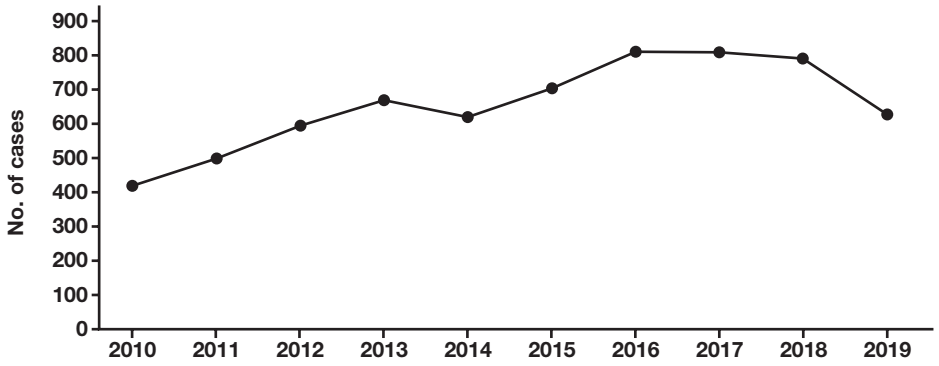
Introduction

Head and neck cancer is the sixth most common cancer in the world.[1] Laryngeal cancer (LC) is the most common malignancy of the head and neck, accounting for 30% to 40% of the head and neck cancer pathology.[2] Histopathologically, LC refers mainly (95%-98%) to squamous cell carcinomas.[3] A higher incidence of LC is prevalent among men compared with women.[3] LC is generally associated with patterns of alcohol, tobacco consumption, and cigarette smoking.[4] Human papillomavirus, Epstein-Barr virus, family history of malignancy, and inherent genetic susceptibility can be associated as risk factors for the induction of a certain proportion of LC.[3]

Globally, the incidence of LC depicts varying patterns. It is the ninth most common cancer in Asia.[5] The incidence of LC is currently on the rise in Sri Lanka, as illustrated in **FIGURE 1**, based on the statistics extracted from the Sri Lanka Cancer Registry from 2010 to 2019.

Despite its prevalent incidence, there has been no significant improvement for LC in terms of the 5-year relative survival rates. Diagnosis of LC in the early stages would present a higher probability of curability.[6] The stage at which diagnosis of the LC is made strongly determines the survivability of the patient.[7] Early diagnosis promotes early treatment and better prognosis. However, the diagnosis of this malignancy generally occurs at the more advanced stages due to the nonspecific nature of symptoms. Risk of recurrence and compromised curability and survivability are characteristic of late diagnosis of LC.[8,9] Aggravating this situation further is the lack of screening programs and guidelines developed particularly for LC.[10] Thus, there exists a strong need to develop a simple yet efficient, noninvasive, and inexpensive test with wide accessibility in primary care settings to enable early screening of LC in asymptomatic individuals who are at high risk for

FIGURE 1. **Incidence pattern of laryngeal cancer in Sri Lanka (Sri Lanka Cancer Registry, 2010-2019).**



the development of LC to reduce diagnostic delays.[10] Hence, attention is directed towards serum biomarkers in which manifestation occurs prior to the onset of malignancy and therefore holds significant value in early cancer detection.[11]

Currently, only a few US Food and Drug Administration–approved tumor-associated serum biomarkers, primarily tumor-associated antigens (TAA), are available.[12] These include HER2/neu for breast cancer, CA125 for ovarian cancer, carcinoembryonic antigen for colon cancer, alpha-fetoprotein for liver cancer, CA19-9 for pancreatic/gastrointestinal cancer, and prostate-specific antigen (PSA) for prostate cancer.[12-14]

However, there are various limitations associated with these tumor antigen biomarkers, particularly in terms of inadequate specificity and sensitivity for the early detection of cancer.[15] The screening of asymptomatic patients is done only using PSA, whereas the applications of other TAAs are limited to the monitoring of disease progression in their respective cancers.[12] Therefore, more attempts are being made towards the identification of potential candidates for novel serological cancer biomarkers with specific focus on tumor-specific constituents that are released into the circulation or body fluids for early detection of cancer.[12]

On exposure to tumor antigens, the immune system elicits immune responses against these autologous proteins through the stimulation of a subset of B cells known as B1 cells (CD5+ B cells) leading to the generation of immunoglobulins known as autoantibodies (AAbs).[16,17] These AAbs present potential as serological biomarkers for early cancer diagnostics.[18]

Several characteristics enable serum AAbs to serve as indispensable biomarkers for early tumor detection[19]: AAbs are either generally absent or present in low levels in normal individuals and in individuals with noncancer conditions. Therefore, they can be used specifically as biomarkers for cancer immunodiagnosis.[20] As the production of AAbs occurs several months or years prior to the manifestation of clinical signs and symptoms of tumorigenesis,[21] they serve as indicators of tumorigenic abnormal cellular processes[22] before the observation of other biomarkers or phenotypic aberrations,[23] enabling the detection of cancer in its early asymptomatic stages or even as early as 5 years prior to the onset of the malignancy.[17]

AAbs are easily accessible for screening through methods such as blood collection, which is of minimal invasiveness, as AAbs produced against TAAs are available in the sera of cancer patients.[16,24] Furthermore, the availability of AAbs in body fluids further allows a more personalized form of tumor monitoring.[17]

Inherent stability as well as persistence of AAbs in circulation for relatively longer periods of time, even after the corresponding tumor antigens are no longer available, is another characteristic that contributes to AAbs being advantageous as biomarkers. This can be attributed to limited proteolysis as opposed to other biomarkers such as TAAs, which may display transient secretion and rapid degradation or clearance after brief circulation.[12] AAbs also display easy detectability compared with TAAs. This is due to the prevalence of a higher concentration of AAbs than their corresponding antigens[19] as a result of AAb amplification by the immune system in response to a single antigen.[18]

Additionally, AAbs possess long half-lives that range from 7 days[17] up to 30 days in circulation,[19] enabling simplified sample collection due to the minimization of hourly fluctuations,[17] and are more stable outside the body than other biomarkers.[19] Furthermore, their detection does not require costly and complex technologies.[18]

The presence of AAbs has been demonstrated in various types of cancers, including breast,[25] lung and small–cell lung,[26] gastrointestinal,[27] ovarian,[24] colorectal,[28] esophageal,[29] hepatocellular, [30] and prostate[31] cancers. AAbs against p53 have recently been presented as useful biomarkers for early diagnosis of colorectal, lung, breast, ovarian, and cervical cancers, among others.[32-35] However, no investigations have been conducted with specific focus on LC.

Thereby, this study aimed to detect anti-LC AAbs among LC patients, to correlate patient AAb levels with different stages of disease and other clinical factors, followed by an investigation of the role of anti-LC AAbs as biomarkers in the prediction and early detection of LC, to promote patient curability and survivability while ensuring good quality of life.

Methods

Study Population

Histologically confirmed LC patients (n = 30) of ages 20 years and above were recruited from the District General Hospital, Chilaw, Sri Lanka. Patients were enrolled from December 2021 to May 2022. Blood samples of a volume of 2 mL were collected from each patient. Demographic and clinical data was recorded using a structured interviewer-administered questionnaire. Blood samples (2 mL) for the control group were obtained from age- and gender-matched individuals without cancer diagnosis.

A volume of 2 mL of blood was drawn from each patient using sterile venipuncture by trained medical professionals of District General Hospital, Chilaw, into sterile blood collection tubes. The blood samples were

allowed to clot at 37°C, and serum was separated by centrifugation at 503*g* for 10 min at 4°C. Sera was stored at –20°C until the conduction of enzyme-linked immunosorbent assay (ELISA).

Ethics Approval

Ethical approval for the study was granted by the Ethics Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka (EC-21-058). Patients were recruited to the study on informed and written consent.

Laryngeal Cancer Tumor Antigen Preparation

The cell line UPCI: SCC152 (American Type Culture Collection [ATCC] CRL-3240), which comprises cells of squamous cell carcinoma of the laryngopharynx, was cultured to extract LC tumor antigens for indirect ELISA. The cell line was cultured to 80% confluency in complete growth medium (Eagle's minimum essential medium with L-glutamine, nonessential amino acids, sodium bicarbonate, fetal bovine serum, glutamate, penicillin-streptomycin, and distilled water) as per instructions specified by the handling information under detailed ATCC product information of UPCl: SCC152, under strict aseptic conditions. The cells were cultured in 1 t-25 flask (25 cm^2) and 1 t-75 flask (75 cm^2).

On reaching 80% confluency, the old culture medium of the cells was discarded and the cells were rinsed 3 times with 3-5 mL of ice-cold phosphate-buffered saline (PBS). The cells were then subjected to trypsinization using trypsin-EDTA solution (1.5 mL for t-25, 2-3 mL for t-75) and incubated at 37°C for 5 min for complete cell detachment. This was then followed by centrifugation at 36*g* at 4°C for 7 min. The cell pellet obtained was resuspended in radioimmunoprecipitation assay (RIPA) cell lysis buffer (190 µL for t-25, 570 µL for t-75) and sonicated for three 1-minute pulses (50% power) while on ice. This was followed by centrifugation at 14,000*g* for 15 min to pellet out the cell debris. The supernatant (crude antigen extract) was stored at –20°C until further use. The 80% confluent t-25 flask produced 190 µL of antigen extract (antigen extract-1) and the t-75 flask produced 570 µL (antigen extract-2).

It is important to note that to ensure efficiency and reproducibility in the preparation of the antigen extracts, the protocol and similar conditions were strictly maintained throughout, including the composition of the RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], and protease inhibitor cocktail [Sigma-Aldrich, P8340] added based on the manufacturer's instructions). Only the volume of the RIPA cell lysis buffer was adjusted according to the size of the flask that the cells were cultured in.

Antigen Quantification

The quantification of the antigenic proteins was performed through the Bradford assay as per the instructions specified in the product information sheet of the Bradford kit (product code: ML106-500 ML, HiMedia). A concentration series in the range of 4 to 40 µg/200 µL was prepared from a stock solution of standard protein, bovine serum albumin (BSA) of a concentration of 1 mg/mL, and the Bradford assay was performed to generate a standard curve of absorbance at 595 nm against the concentration of protein (µg/200 µL). The assay was similarly performed to a tenfold-diluted sample of the extracted antigen as well as a sample of RIPA cell lysis buffer for rectification. Absorbance readings were obtained in triplicates. Using the absorbance readings and the standard curve, the concentration of the antigen extract was determined. The concentration of 190 µL of antigen extract produced from the t-25 flask (antigen extract-1) was estimated as 373.75 µg/mL, whereas that of the 570 µL of antigen extract produced from the t-75 flask (antigen extract-2) was 195.50 µg/mL.

Antigen Visualization

The extracted antigens were subjected to qualitative and quantitative analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), performed using the omniPAGE Mini TETRAD Vertical Protein Electrophoresis System, as per instructions specified in the manual of the Mini-PROTEAN 3 cell-system (catalog number: 165-3301, Bio-Rad). The gel comprised 15% running gel and 5% stacking gel. The prestained protein ladder used was the 10-225 kDa Broad Range Molecular Marker (Promega). BSA samples (1 mg/mL) were used as positive controls. Volumes of 5 µL protein ladder, 10 µL antigen and BSA samples each, were loaded into the wells. Each of the antigen and BSA samples were loaded in 2 different dilutions, 1:1 and 1:2, with sample buffer. The samples diluted in sample buffer were heated at 95°C for 4 min prior to loading.

The SDS-PAGE was run under constant current of 10 mA at 150 V. On completion of the run, the dye front measurement was obtained, followed by fixing (with fixing solution for 30 min on a shaker), staining (with staining solution for 30 min on a shaker) and destaining of the gel (in destaining solution on a shaker overnight). Subsequently, the distance travelled by each protein band was measured and the relative mobility of each of the protein bands was calculated. Finally, a graph of the log molecular weight against relative mobility was derived for the protein ladder. The molecular weights of the unknown proteins that constitute the antigen extracts were determined using the graph.

Detection of Laryngeal Cancer-Associated Autoantibodies

The 96-well flat-bottomed polystyrene microplate was coated with 100 µL of 0.5 µg/mL antigenic proteins diluted in 0.13 M PBS. The plate was then incubated in a humid chamber at 37°C for 2 h, followed by overnight incubation at 4°C. After incubation, the antigens were aspirated and the plate was washed 3 times with washing buffer/PBS with Tween 20 (PBST). The wells were then blocked with 200 µL of blocking buffer (1 × 0.13 M PBS with 5% skimmed milk) and incubated for 1 h at 37°C. After 6 washes, 100 µL from each serum sample diluted to 1:100 in antibody dilution buffer (PBST with 5% skimmed milk) was added. This was followed by incubation at 37°C for 1 h and 15 min. After another 6 washes, 100 µL of goat anti-human IgM (µ-chain specific)-horseradish peroxidase conjugate (MyBioSource), diluted to 1:6000 in dilution buffer (PBST with 5% skimmed milk), was added to the plate and incubated at 37°C for 1 h and 15 min. Following incubation, the plate was washed 6 times and a volume of 100 µL of substrate solution (30, 30, 50, 50-tetramethylbenzidine dissolved in dimethyl sulfoxide, phosphate citrate buffer, and 30% hydrogen peroxide) was added to each well. The plate was then incubated at room temperature in the dark for 30-40 min. The reaction was then stopped by adding 50 µL of 1 M HCl to each well. The optical density (OD) values were then obtained at 450 nm using an ELISA plate reader. The samples were tested in duplicates.

Statistical Analyses

The determined AAb levels (in terms of OD values) and clinical data recorded from each recruited patient, particularly the stage, time duration since first symptom appearance, time duration of medical attention, as

TABLE 1. Demographic characteristics of the study cohort

Demographic characteristics	No. (%)
Gender	
Male	28 (96.7)
Female	2 (6.7)
Age groups, y	
40-50	5 (16.7)
50-60	13 (43.3)
60-70	6 (20.0)
70+	6 (20.0)
Exposure to risk factors	
Alcohol	17 (56.7)
Tobacco	17 (56.7)
Smoking	21 (70.0)
Family history of malignancy	1 (3.3)
No exposure to any risk factors	3 (10)

well as risk factor exposure, were statistically analyzed with SPSS, version 26.0 (IBM).

Independent samples *t*-test (CI = 95%, $P \leq .05$) was performed to determine a significant difference between AAb levels of the patient and control groups.

To evaluate the diagnostic potential of the application of anti-LC AAbs for early detection of LC, 4 different cutoff OD values were produced: mean OD value of healthy controls + SD, mean OD value of healthy controls + 2 SD, mean OD value of healthy controls + 3 SD, and the cutoff obtained from receiver operating characteristic (ROC) curve analysis. The most accurate cutoff value was selected based on the diagnostic accuracies (sensitivity, specificity, positive predictive value [PPV], and negative predictive value [NPV]). Using the selected OD cutoff, the diagnostic potential of the application of anti-LC AAbs for early detection of LC was evaluated by determining indices of diagnostic performance such as sensitivity, specificity, PPV, NPV, and overall accuracy. Additionally, the positive likelihood ratio (LR+) and negative likelihood ratio (LR–) were calculated.

One-way analysis of variance (ANOVA) tests (CI = 95%, $P \leq .05$) were conducted to investigate the variations in AAb levels of patients with clinical factors such as different stages of cancer, different time durations since first symptom appearance, different time durations of medical attention, and different degrees of exposure to risk factors.

Results

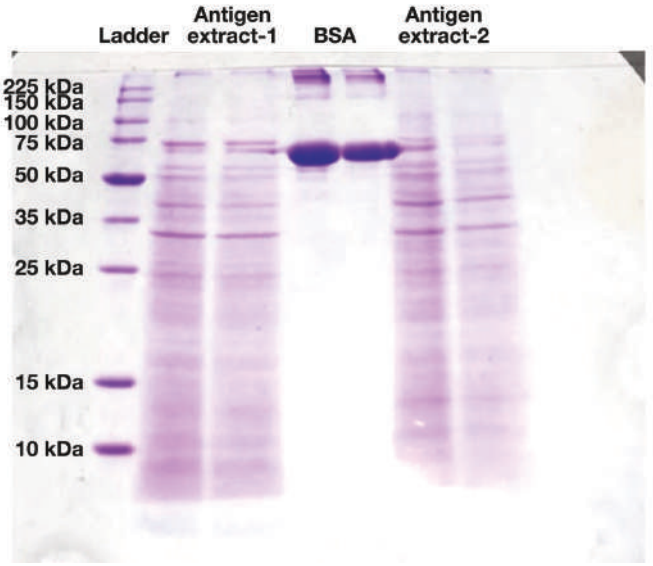
Demographic Analysis of Patient Population

The demographics of the LC patient population are presented in **TABLE 1**.

Antigenic Analysis

The molecular weights of all the characteristic antigen proteins that may be found in a crude antigen extract derived from UPCl: SCC152, ranged approximately from 7 kDa to 143 kDa. Antigens of the molecular weights 42.462 kDa, 56.754 kDa, 67.453 kDa, 71.614 kDa, 85.114 kDa, and 90.157 kDa appeared to be the most prominent. Both antigen

FIGURE 2. Antigen protein visualization by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). BSA, bovine serum albumin.



extracts displayed similar expressions in the pattern of protein bands (**FIGURE 2**), thereby validating the reproducibility of the antigen extract preparation followed in the study.

Autoantibodies in Laryngeal Cancer

The patient samples presented a maximum OD value of 0.282 and minimum OD value of 0.067 with an OD mean ± SD of 0.118 (± 0.054). The healthy controls showed a maximum OD value of 0.175 and a minimum OD value of 0.055 with an OD mean ± SD of 0.090 (± 0.033).

A significant difference between the mean OD values of the patient and control populations in the study was observed (independent samples *t*-test, $P = .019$), indicating both the presence of anti-LC AAbs in sera of LC patients as well as its detectability at significantly high levels in patients compared with healthy individuals (**FIGURE 3**).

Evaluation of Diagnostic Performance of Autoantibody-Level Testing

The OD value of 0.087 obtained from the ROC curve analysis was determined to be the most appropriate cutoff with an area under the curve (AUC) of 0.704 ($P = .007$) (**FIGURE 4**). This cutoff OD value was applied to the OD values of the patient cohort (n = 30) and healthy control cohort (n = 30) to evaluate the diagnostic performance of anti-LC AAb-level testing for the detection of LC (**TABLE 2**).

Associations Between Autoantibody Levels and Different Clinical Factors

AAb levels of patients were determined to not be significantly different among the different stages of cancer (1-way ANOVA, $P = .708$). This implies that a significant level of anti-LC AAbs may be present in LC patients irrespective of the stage, indicating that the level of AAbs prevalent in the patient population at stage I of LC would be just as sufficient as that of the later stages to be detected. Therefore, as AAb detection may also be achieved during stage I of the disease, the utilization of anti-LC AAbs as a biomarker presents promising potential for the early detection of LC.

FIGURE 3. Autoantibodies in patient and control cohorts. OD, optical density.

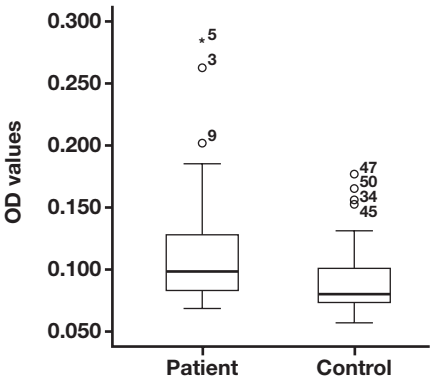
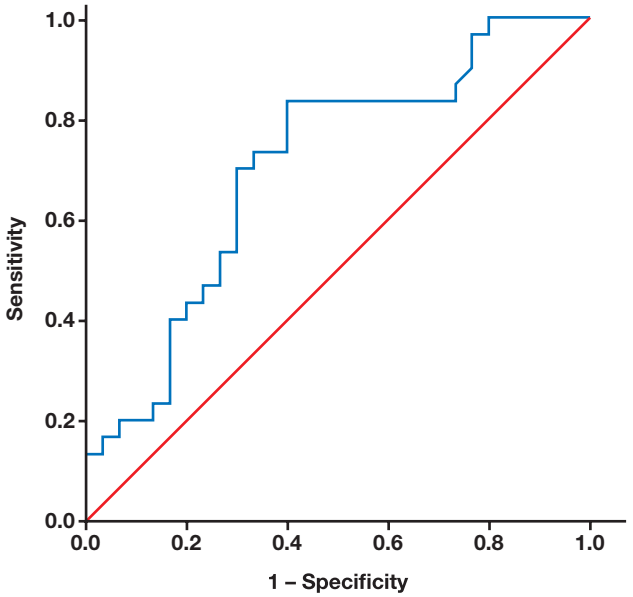


FIGURE 4. Receiver operating characteristic curve analysis.



Additionally, patient AAb levels were found to not vary significantly with different time durations since the first appearance of symptoms (1-way ANOVA, $P = .228$), different time durations of the reception of medical attention (1-way ANOVA, $P = .231$), as well as different degrees of risk factor exposure (1-way ANOVA, $P = .478$).

Discussion

This study demonstrated the presence of anti-LC AAbs and their potential as diagnostic biomarkers for the early detection of LC. However, diagnostic accuracy, especially in terms of sensitivity and specificity, each of which was determined to be 70%, could be improved further.

False negatives for AAb detection may arise due to tumor heterogeneity and diversity in immune responses of the patient population, which results in variations of patient AAb levels. This could be attributed to biological complications such as the natural inhibition of AAb generation against TAAs[36] as well as the variability in tumor immunogenicity.[37] Different TAAs may be immunogenic in different patients with the same cancer, and the AAb responses induced within patients having the same

TABLE 2. Diagnostic performance of autoantibody-level testing for the cutoff OD_{450} 0.087 in the detection of laryngeal cancer

	Diagnostic performance for the cutoff, OD_{450} 0.087
Laryngeal cancer patients (n = 30)	
Positive	21
Negative	9
Healthy control individuals (n = 30)	
Positive	9
Negative	21
Sensitivity (%)	70.0
Specificity (%)	70.0
PPV (%)	70.0
NPV (%)	70.0
Accuracy (%)	70.0
LR+	2.33
LR–	0.43

LR–, negative likelihood ratio; LR+, positive likelihood ratio; NPV, negative predictive value; PPV, positive predictive value

cancer may occur against different sets of antigens.[14,17,38] The expression of AAbs against a particular TAA may only be observed in 10% to 30% of patients.[39,40] Therefore, in the study, different patients would express different AAb levels based on the presence of the types of LC antigens in the extract. Furthermore, patient's gender may influence AAb levels. Female hormonal factors are considered to have a protective role in cancer, allowing better survivability in female patients than males.[41] It is assumed that beta-estradiol and progesterone in female patients slow down tumor progression. This may lead to comparative reductions in TAAs and, thereby, the level of AAbs elicited in female patients.

False positives may be attributed to the occasional presence of naturally occurring AAbs in normal individuals under certain circumstances, particularly in older individuals,[14,42] that result in nonspecific reactivity of natural AAbs.[17]

Low sensitivity and specificity remain major issues that hinder the clinical application of most identified tumor AAbs. Nevertheless, there has been an increase in identification of AAbs that possess improved sensitivity and specificity. Moreover, through the combination of potential AAbs to form a diagnostic biomarker panel, improved sensitivity and specificity scores can be achieved. For instance, O'Rouke et al[43] tested the diagnostic performance of a biomarker panel compared against that of PSA alone for the diagnosis of prostate cancer. An increase in sensitivity from 12.2% to 95% was achieved through the biomarker panel, whereas specificity remained at 80% for both PSA and the biomarker panel. Similarly, the development of AAb panels for LC could improve sensitivity and specificity in its detection. However, this may be challenged by the lack of defined tumor antigens that are implicated in LC.[44] Discovery of tumor-specific antigens unique to LC would aid in enhancing the diagnostic performance of anti-LC AAbs for the detection of LC. Future studies are required to identify such tumor antigens in LC.

Because the availability of known tumor-specific antigens are few to none for LC, this study was limited to the utilization of whole cell crude antigen preparations to ensure optimal exploitation of immune reactivity between the different tumor antigens implicated in LC and AAbs in patient sera. The unknown constituent antigens of the crude

extract remain to be identified. This investigation was able to analyze and determine the approximate molecular weights of these unknown antigen constituents, which are postulated to be characteristic tumor antigens involved in LC. The research could be expanded to isolate and identify LC-specific antigens present in the crude extract using Western blotting. Antigen preparation protocols could be conditioned, targeting toward the separation and identification of different types of antigens in LC including cell membrane, nuclear, and secreted antigens. This research potentially directs future investigations toward a long-term goal of isolation and identification of LC-specific antigens.

Future research should focus on specific identification of LC-associated tumor antigens and anti-LC AAbs to develop AAb panels of high diagnostic accuracy to aid in the early diagnosis of LC. This would ensure appropriate therapeutic interventions targeting early-stage LC, which would lead to more successful patient outcomes.

As this study was conducted on a pilot scale with a smaller sample size, further studies are required that use larger cohorts to avoid intersample variations and to validate the obtained results.

Conclusion

This study revealed that AAbs may be detected at significant levels in LC patients irrespective of cancer stage—even at stages as early as stage I—with good diagnostic accuracy. However, as the study was conducted on a pilot scale, further validation with a larger sample size is required. The findings of the investigation are reflective of the potential of anti-LC AAbs as predictive biomarkers for early diagnosis of LC, which may contribute to improved curability and survivability of LC patients while maintaining their quality of life. Important future directions of the study include isolation and identification of LC-specific antigens, which would lead to the identification of LC-specific AAbs and, thereby, the development of AAb biomarker panels for the early detection of LC with improved diagnostic accuracy.

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Conflict of Interest Disclosure

The authors have nothing to disclose.

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Exploratory analysis of glial fibrillary acidic protein and ubiquitin C-terminal hydrolase L1 in management of patients with mild neurological symptoms undergoing head computed tomography scan at the emergency department: a pilot study from a Croatian tertiary hospital

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Key words: mild traumatic brain injury; biomarkers; glial fibrillary acidic protein; ubiquitin C-terminal hydrolase L1; diagnostic accuracy; computed tomography

Abbreviations: GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase L1; ED, emergency department; mTBI, mild traumatic brain injury; CT, computed tomography

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ABSTRACT

Background: Diagnostic accuracy of glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase L1 (UCH-L1) in identification of intracranial abnormalities detected by computed tomography (CT) in mild traumatic brain injury (mTBI), and in patients with mild neurological symptoms not caused by head trauma but suspected with a neurological disorder, was examined.

Methods: GFAP and UCH-L1 were determined using the chemiluminescence immunoassays on the Alinity i analyzer (Abbott Laboratories).

Results: Significantly higher GFAP (median 53.8 vs 25.7 ng/L, $P < .001$) and UCH-L1 (median 350.9 vs 153.9 ng/L, $P < .001$) were found in mTBI compared to non-head trauma patients. In mTBI diagnostic sensitivity (Se) and specificity (Sp) for the combination of GFAP and UCH-L1 were 100% and 30.9%, respectively, with area under the curve (AUC) 0.655. GFAP alone yielded Se 85.7%, Sp 41.8%, and AUC 0.638, while UCH-L1 yielded Se 57.1%, Sp 56.4%, and AUC 0.568. In non-head trauma patients, the combination of GFAP and UCH-L1 showed Se 100%, Sp 87.9%, and AUC 0.939, while GFAP alone demonstrated Se 100%, Sp 90.9%, and AUC 0.955.

Conclusions: If these results are reproduced on a larger sample, GFAP and UCH-L1 may reduce CT use in patients with mild neurological symptoms after systemic causes exclusion and neurologist's evaluation.

Introduction

Mild traumatic brain injury (mTBI) is the most common neurological disorder that affects up to 80% of all patients who have experienced a sudden head trauma. Its diagnosis is established based on a recent head trauma that causes loss of consciousness lasting no more than 30 minutes and mild neurological symptoms, and is graded by the Glasgow Coma Scale (GCS) scores between 13 and 15.[1-3] Patient management largely occurs at the emergency department (ED), which is often the only point of medical contact for patients suspected with mTBI.[3] Despite being the least severe of all brain injuries, traumatic intracranial lesions might be found in 10%-20% of patients, thus requiring immediate attention and prompt intervention.[4-6] Therefore, computed tomography (CT) head scan, which represents the diagnostic standard for detection of intracranial abnormalities at the ED, is performed regularly in all patients with suspected mTBI. However, anamnestic data might be ambiguous, since many patients cannot reliably recall experiencing a head trauma, a body jolt, or blow that caused brain jostling, making initial patient triage challenging. Thus, CT is performed also in the majority of patients presenting at the ED with a complaint of mild neurological symptoms, regardless of a known positive history of head trauma.[6,7] In addition, mild and nonspecific symptoms commonly observed as a consequence of mTBI, such as headache, vertigo and nausea, can be encountered in a variety of other

pathophysiological conditions, either of neurological origin or secondary to nonneurological disorders. Therefore, in order to exclude neurological conditions as a cause of these symptoms in patients presenting at the ED, head CT scan is commonly performed.[7,8] Surely, this diagnostic approach implies patient radiation exposure, prolongs patients' stay at the ED, and poses a significant burden on ED physicians.[6,9] In addition, nonselective use of CT generates significant unnecessary costs given that only a small rate of patients with mild neurological symptoms eventually have intracranial abnormalities.[9] Therefore, there is a clear clinical need for identifying objective and minimally invasive, early blood-based biomarkers that reflect minor neuronal damage and in that way reduce the need for CT and optimize patient diagnostic evaluation at the time of presentation to the ED.[10]

Combined measurement of glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase L1 (UCH-L1) within 12 hours from head injury was cleared by the United States Food and Drug Administration in 2018 for clinical use in adult patients with mTBI to help determine the need for CT.[6] Given the heterogeneous case mix of patients with mild neurological symptoms of unknown origin that overlap with those encountered in mTBI as well as ambiguous anamnestic data at admission to the ED, it would be valuable also to explore their utility in predicting or excluding CT abnormalities in patients presenting with mild neurological symptoms not known to be caused by a head trauma.

The aim of the present study was to assess the diagnostic accuracy of GFAP and UCH-L1, both individually and in combination, in identification of intracranial abnormalities detected by CT in patients with mTBI. In addition, we aimed to evaluate the diagnostic accuracy of the TBI biomarkers in relation to head CT scan in patients presenting at the ED with mild neurological symptoms not caused by a head trauma in whom a neurological disorder was suspected after being evaluated by a neurologist.

Methods

Study Setting and Participants

This single-center, cross-sectional study was conducted from May 2022 to March 2023 at the University Hospital Center Zagreb. This is the largest tertiary academic hospital in Croatia that serves most of Central and Northern Croatia for specialist and acute medical procesures. It consists of 28 medical departments with 1800 beds and 5500 employees and provides general and advanced medical care in all fields, including emergency medicine, intensive care, general surgery, neurosurgery, orthopedic surgery, anesthesiology, radiology, internal medicine, specialized intensive care, oral, plastic and maxillofacial surgery as well as all other medical specialties that would qualify it as a US level I trauma center.

Study participants were recruited at the Department of Emergency Medicine, which provides acute care and treatment for around 110,000 patients annually. It provides emergency medical care in the fields of internal medicine, neurology, surgery, ophthalmology, urology, pediatrics, pulmology, anaesthesiology and otorhinolaryngology, with dedicated units for each medical specialty. The ED team consists of senior medical doctors and residents of different medical specialties, nurses and medical technicians as well as a number of specially trained ED doctors with completed 5-year residency in emergency medicine.

All laboratory analyses were performed at the emergency laboratory of the Department of Laboratory Diagnostics which is located within the premises of the ED and provides laboratory services exclusively for outpatients admitted to the ED. Such proximity of the dedicated laboratory to the ED implies the shortest possible turnaround time for receiving laboratory results, thus contributing to the efficiency and timeliness of the patient management at the ED.

The study included 62 adult patients who were classified as having mTBI based on an acute head trauma event that happened within 12 hours before admission and was commonly followed by mild neurological symptoms including headache, vertigo, nausea, vomiting, alterations in mental status such as confusion, amnesia, short-term loss of consciousness (< 30 minutes) and/or disorientation. Patients with older head trauma, those whose history included any kind of known neurological or psychiatric disorders, neurosurgical interventions, and/or previous traumatic brain lesions confirmed by CT were excluded. Since approximately 1 to 2 patients with mTBI present daily to our ED, yielding roughly 500 patients in the study timeframe, 12% of total number of patients with mTBI were enrolled for the purposes of this study.

In addition, 46 patients who presented with the same symptoms but who did not experience an acute brain injury were enrolled. Only patients in whom, following a medical check-up, a neurological disorder was suspected and to whom subsequently a head CT scan was indicated by the attending physician and the consultant neurologist at the ED, were included. Patients in whom clinical and laboratory evaluation revealed that the presenting symptoms are a consequence of an underlying gastrointestinal disease, sepsis, pneumonia, or any other confirmed nonneurological disorder were not included in the study. We assessed a selected group of neurology patients presenting at the ED with mild neurological symptoms who underwent head CT scan after they were screened in the ED by a neurologist and after systemic causes were excluded.

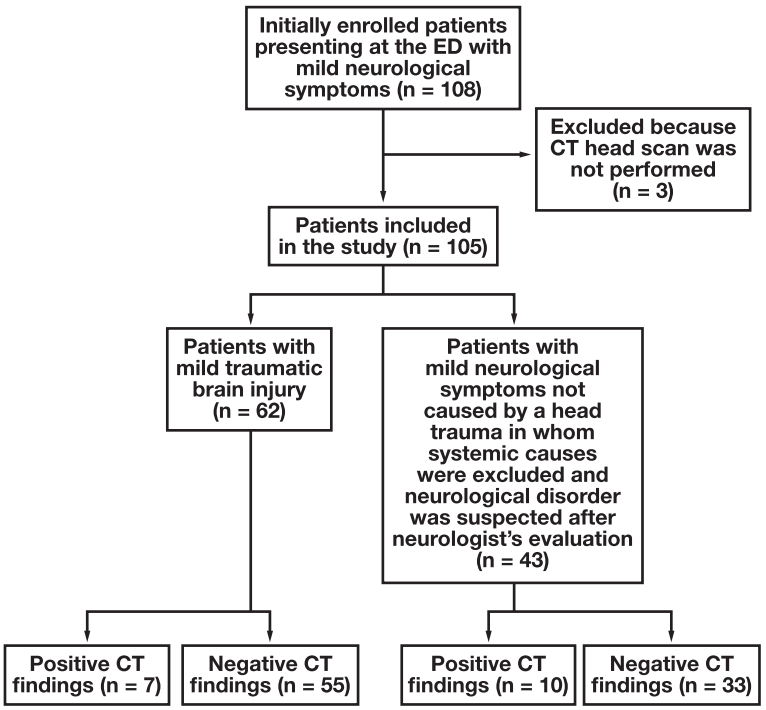
All enrolled patients had a GCS score between 13 and 15 at the time of presentation to the ED. Patients were enrolled prospectively at the time of admission to the ED and the attending physician decided about their inclusion in the study, based on their anamnesis and results of clinical examination. Eligibility for inclusion in the study was further confirmed independently by another member of the research group.

Patient management included blood sampling for routine laboratory diagnostics, CT head scan, clinical examination, and calculation of the GCS score.

Laboratory Measurement of GFAP and UCH-L1

GFAP and UCH-L1 were determined in blood samples that were obtained for routine laboratory testing during the medical visit at the ED. Blood that had already been drawn for clinical purposes was used to measure GFAP and UCH-L1. Therefore, samples were handled by the laboratory before being identified for the study. There may have been a delay before samples were analyzed. Blood was drawn into 3-mL VACUETTE tubes (Greiner Bio-One) containing lithium heparin as the anticoagulant. Samples were without delay delivered to the emergency laboratory and were processed completely adhering to manufacturer's instructions.[11] Specifically, plasma for testing was obtained by 10 minute centrifugation at 2100*g* within a maximum of 1 hour from blood draw. Plasma was immediately separated from cells, aliquoted in clean polypropylene microtubes (Eppendorf) and frozen at –20 °C for a maximum of 1 month. Prior to analysis, plasma was thawed, mixed using a vortex and recentrifuged in order to obtain plasma homogeneity. To confirm that the sample is homogeneous and to exclude the possible presence of clots, fibrin threads, or other solid particles that could interfere with measurement and yield unreliable results, plasma was also

FIGURE 1. **Flow diagram of the patient selection and classification process. CT, computed tomography; ED, emergency department.**



visually inspected prior to analysis and, if necessary, centrifugation was repeated. GFAP and UCH-L1 were measured using the automated chemiluminescent microparticle immunoassays applied on the Alinity i analyzer (Abbott Laboratories). As defined by the manufacturer, the cut-off value for GFAP is 35.0 ng/mL and for UCH-L1 is 400.0 ng/mL. This TBI test panel is considered as positive if either of the 2 biomarkers is above the predefined cut-off.

The study was conducted completely according to the Declaration of Helsinki and was approved by the University Hospital Center Zagreb Ethics Committee (number of approval: 8.1-23/38-2, 02/013 AG) and all patients signed an informed consent prior to study enrollment.

Statistical Analysis

Data distribution normality was assessed using the Shapiro-Wilk test. The difference of GFAP and UHC-L1 results between patients with and without head trauma was tested using the Mann-Whitney test, with $P < .05$ considered as statistically significant. Correlation between GFAP and UCH-L1 was assessed with the Spearman's rank correlation coefficient (ρ). Diagnostic accuracy of GFAP and UCH-L1 in identification of CT abnormalities was determined using receiver operating characteristic (ROC) analysis. The area under the curve (AUC) and diagnostic sensitivity and specificity with corresponding 95% CI were calculated. Post-hoc power analysis for the included number of mTBI patients was performed using the 7% reference incidence of positive CT findings in mTBI obtained in the study by Papa et al.[6] The obtained power was 28% (α of 0.05). Statistical analysis was performed using MedCalc software, version 19.5.2.

Results

Out of 108 initially enrolled study participants, 3 patients without head trauma eventually did not undergo a CT head scan and were therefore excluded from the study. Head CT scan revealed intracranial abnormalities in 17 (16%) patients. Flow diagram of patient inclusion and classification is shown in **FIGURE 1**. **TABLE 1** presents demographic and clinical data of the included patients.

Statistically significant higher values of both GFAP and UCH-L1 were obtained in patients with head trauma compared to those without head trauma (**FIGURE 2**). Weak correlation between GFAP and UCH-L1 was obtained, with Spearman's ρ being 0.372 (95% CI: 0.194-0.526).

GFAP was below the predefined cut-off in 24/62 mTBI patients and 34/43 patients without head trauma, while UCH-L1 was below its cut-off in 30/62 patients with mTBI and in 42/43 non-head trauma patients. All patients with positive CT findings had positive interpretation of the TBI test panel. When evaluated individually, GFAP was above the predefined cut-off in 6/7 patients with head trauma and positive CT and in all 10 patients without head trauma and positive CT. UCH-L1 was elevated in 4/7 patients with head trauma and positive CT, and in only 1 patient of the total 43 patients without head trauma. Diagnostic accuracy data of the single and combined use of GFAP and UCH-L1 in identification of intracranial abnormalities confirmed by CT in mTBI patients, patients without head trauma, and all included patients regardless of head trauma are presented in **TABLE 2**.

Discussion

The present exploratory analysis of the TBI dual biomarker test panel comprising GFAP and UCH-L1 in the management of patients with mild neurological symptoms with and without an underlying head trauma presenting at the ED seemed to discriminate between those with a positive and negative CT.

Both GFAP and UCH-L1 were significantly higher in patients with mTBI compared to patients presenting with mild neurological

TABLE 1. Demographic and Clinical Data of Included Study Participants With and Without Head Trauma[a]

	Patients with head trauma (n = 62)	Patients without head trauma (n = 43)
Age, y, median (range)	62 (18-96)	57 (18-85)
Male	28 (45)	11 (26)
Time from head injury to presentation to the ED, h, median (range)	2 (0.5-8)	N/A
Time from head injury to blood draw, h, median (range)	2.5 (1-10)	N/A
Mechanism of head injury		N/A
Fall	57 (92)	
Car accident	5 (8)	
Glasgow Coma Scale score, median (range)	15 (13-15)	15 (14-15)
Hospitalized patients	20 (32)	4 (9)
Patients hospitalized at the ICU	4 (6)	0 (0)
Positive CT findings	7 (11)	10 (23) Acute ischemic lesions (3/10): acute ischemic stroke (2/3), transient ischemic attack (1/3) Arachnoid cyst (2/10) Cerebral microangiopathy (2/10) Cerebral atrophy with inconclusive final neurological diagnosis (1/10) Hypertensive hydrocephalus (1/10) Meningioma (1/10)
Subdural hematoma	(3/7)	
Epidural hematoma	(1/7)	
Intracerebral hematoma	(1/7)	
Periorbital hematoma	(1/7)	
Subarrachnoid hemorrhage	(1/7)	
Patients with more than 1 clinical sign or symptom	16 (26)	7 (16)
No specific clinical signs or symptoms	16 (26)	0 (0)
Pain in shoulder/arm/back	15 (24)	0 (0)
Headache	12 (19)	31 (72)
Head hematoma	10 (16)	0 (0)
Nausea	8 (13)	4 (9)
Vomiting	8 (13)	1 (2)
Vertigo	3 (5)	19 (44)
Shortness of breath	3 (5)	0 (0)
Loss of balance	2 (3)	0 (0)
Short-term loss of consciousness (< 30 min)	2 (3)	0 (0)

CT, computed tomography; ED, emergency department; ICU, intensive care unit; N/A, not applicable.
[a]Data are given as No. (%) except where noted.

FIGURE 2. Comparison of glial fibrillary acidic protein (GFAP) (A) and ubiquitin C-terminal hydrolase L1 (UCH-L1) (B) values between patients with and without head trauma. Results are presented as medians and interquartile ranges; *P* < .05 is considered statistically significant.

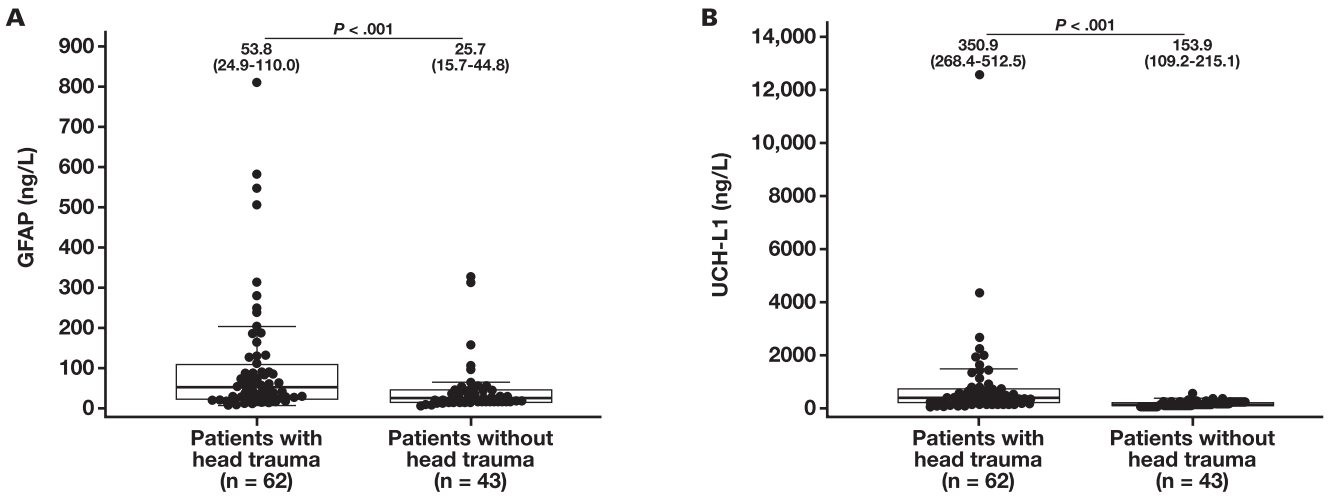


TABLE 2. Diagnostic Accuracy Data of GFAP and UCH-L1, Individually and in Combination

Parameter	AUC (95% CI)	Diagnostic sensitivity, % (95% CI)	Diagnostic specificity, % (95% CI)
Patients with head trauma (n = 62)			
GFAP	0.638 (0.506-0.756)	85.7 (42.1-99.6)	41.8 (28.7-55.9)
UCH-L1	0.568 (0.436-0.693)	57.1 (18.4-90.1)	56.4 (42.3-69.7)
Combination of GFAP and UCH-L1	0.655 (0.523-0.771)	100 (59.0-100)	30.9 (19.1-44.8)
Patients without head trauma (n = 43)[a]			
GFAP	0.955 (0.843-0.995)	100 (69.2-100)	90.9 (75.7-98.1)
Combination of GFAP and UCH-L1	0.939 (0.822-0.989)	100 (69.2-100)	87.9 (72.0-96.6)
All patients (n = 105)			
GFAP	0.772 (0.680-0.848)	94.1 (71.3-99.9)	60.2 (49.2-70.5)
UCH-L1	0.476 (0.377-0.575)	23.5 (6.8-49.9)	71.6 (61.0-80.7)
Combination of GFAP and UCH-L1	0.761 (0.668-0.839)	100 (80.5-100)	52.3 (41.4-63.0)

AUC, area under the curve; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase L1.
[a]In this group, only one patient had elevated UCH-L1, therefore receiver operating characteristic analysis could not be reliably performed.

symptomatology not caused by an underlying head trauma but in whom a causative neurological disorder was suspected. However, overlapping values and their weak correlation indicate different kinetics and release patterns into the bloodstream that are not exclusively related to mTBI.

ROC analysis yielded 100% diagnostic sensitivity of the combined use of GFAP and UCH-L1 in identification of intracranial abnormalities in mTBI patients, which is in accordance with previously published data.[6,9,12] Similarly to previous studies, low diagnostic specificities for both the combined and single use of GFAP and UCH-L1 were obtained in patients with mTBI, indicating that even subtle brain injuries not causing CT-evident intracranial lesions can result in elevations of either of the biomarkers.[6,9] As evidenced by higher diagnostic sensitivity and AUC, measurement of GFAP alone in patients with mTBI in our study cohort outperformed UCH-L1 for the purpose of exclusion of CT-detectable intracranial abnormalities, which is consistent with findings from previously published studies.[6,13] However, all previously conducted studies uniformly revealed only moderate discriminatory ability of GFAP and UCH-L1 when used alone, as also confirmed herein. Some heterogeneities of the reported diagnostic accuracy data among studies are present and can be attributed to the different origin and kinetics of GFAP and UCH-L1 following brain injury, but also to diversity of undertaken study designs. In our study, markedly lower AUCs were obtained. Data variability might be a consequence of including patients with different degrees of brain injury severities and associated neurological symptoms, of patient recruitment at different timepoints following brain injury, nonuniformity of criteria for mTBI diagnosis, possible variabilities in sample processing and storage, as well as use of different immunoassays and cut-offs.

Another finding of this study is that the combination of GFAP and UCH-L1 did not yield a single false-negative interpretation in patients with mild neurological symptoms not caused by an underlying head trauma who were suspected of having an underlying neurological disorder and in whom intracranial abnormalities were detected by CT. These results performed in 43 patients suggest that a negative test panel of GFAP and UCH-L1 might reduce the use of CT in patients with mild neurological symptoms not caused by head trauma. Besides 100% diagnostic senstivity, superior diagnostic specificity of the evaluated test panel was obtained in this group of patients compared to those with an underlying head trauma. The main contributor to such impressive discriminatory ability of the TBI test panel was GFAP. In fact, since UCH-L1 yielded 1 additional false-positive result and therefore led to 1 more false-positive interpretation of the TBI test panel, GFAP alone even outperformed its combined use with UCH-L1, yielding 100% diagnostic sensitivity, diagnostic specificity over 90%, and AUC of 0.955. Of note, these patients were all evaluated by a neurologist before determining their eligibility and were evaluated to ensure that other possible systemic causes were excluded. If these findings can be reproduced in a much larger sample of patients, negative GFAP values alone or in combination with negative UCH-L1 may help reduce CT use in patients with mild neurological symptoms not caused by an underlying head trauma. Moreover, elevated GFAP values in such patients should raise suspicion on the presence of any kind of brain lesion and the patient should be directed to further diagnostic management. These results confirm that although being brain-specific, GFAP is not disease-specific and can be released into the bloodstream in a wide variety of conditions other than mTBI that cause neuronal damage, including cerebrovascular disorders, as well as both benign and malignant processes of different etiology within the central nervous system.[14] On the contrary, none of the latter neurological disorders in patients without head trauma were characterized by UCH-L1 elevations, at least not above the predefined threshold of 400 ng/L that is intended for mTBI assessment.

This study has several limitations that affect the reliability of the obtained results. Firstly, the sample size is not adequately powered to draw reliable conclusion. Along with the overall low number of study participants, the study included only several patients with abnormal CT findings, which might produce results that cannot be fully translated to reflect the general population. Therefore, additional investigations on a larger patient cohort are necessary. Secondly, the timing from head injury or symptoms onset was based exclusively on either patients' personal statements or was reported by their accompanying person, thus being prone to subjective perception. Also, more precise information regarding the timeframe from head injury to patient admission as well as blood draw would be useful in order to gain insight into GFAP and UCH-L1 release kinetics. Moreover, longitudinal monitoring of diagnostic accuracy at several timepoints following head injury or neurological symptoms onset would elucidate the changing pattern of GFAP and UCH-L1 over time and in that way provide basis for more

accurate results interpretation. Finally, a more evidence-based approach in selecting patients who need to undergo CT head scan in the first place according to an expert panel opinion and thorough clinical evaluation would provide more reliable and applicable study results. Due to all these limitations—and also significantly lower AUC in mTBI patients when compared to previously published data, which raises suspicion on improper sample processing and storage, hence possibly causing altered analyte stability and consequently yielding the observed discrepancies—the presented results should be considered exclusively as preliminary. Importantly, the obtained results should be interpreted with caution and the study should be considered only as an exploratory analysis that requires continuation of research, with inevitable fine tuning of study design and patient selection protocol. Nevertheless, our study reflects a real-life case mix of patients that undergo CT head scan at the ED due to mild neurological symptoms, the number of whom is increasing rapidly but is not always supported by firm clinical indication.[15] Subsequently, such a nonrational diagnostic approach poses a risk that incidental and nonspecific findings not related to the initial clinical question will be detected, as also evidenced in the present study. The authors recognize that many patients who present to the ED with mild neurologic symptoms do not have a neurologic disease. These symptoms may be signs of systemic conditions such as infection, sepsis, coronary artery disease, and gastrointestinal disease. This is why all our patients were evaluated for systematic diseases and by a neurologist at the ED.

In conclusion, if these results can be reproduced in a much larger sample of patients, GFAP and UCH-L1 measurement may be used to reduce CT use in patients with mild neurological symptoms after having systemic causes excluded and an evaluation by a neurologist.

However, due to the nonspecificity of mild neurological symptoms that can be present in a wide variety of pathophysiological conditions of nonneurological origin, it is of upmost importance to properly triage the patients based on their clinical presentation prior to laboratory ordering of GFAP and UCH-L1. Finally, due to possible elevations of both GFAP and UCH-L1 not caused exclusively by brain pathology, positive findings need to be interpreted with caution and in conjuction with clinical data. Further large-scale studies including patients with diverse neurological conditions are needed for an objective judgement on the utility and practical applicability of GFAP and UCH-L1 measurement in the diagnostic management of patients both with and without recent head trauma who present at the ED with mild neurological symptoms.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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The role of gastrin 17 and pepsinogen I:pepsinogen II ratio in pathological diagnosis and endoscopic selection in gastritis patients

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Key words: gastritis; gastrin-17; pepsinogen I; pepsinogen II; colonoscopy; clinical pathology; AP gastrointestinal

Abbreviations: PGI, pepsinogen I; PGII, pepsinogen II; G17, gastrin-17; PGR, PGII:PGII ratio; OLGA, operative link on gastritis assessment; OLGIM, operative link for gastritis intestinal metaplasia assessment; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; ROC, receiver operating characteristic; AUROC, area under the ROC curves

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ABSTRACT

Background: The noninvasive serum markers pepsinogen I (PGI), pepsinogen II (PGII), gastrin-17 (G17), and PGI:PGII ratio (PGR) have recently been proposed as a new tool for predicting various gastric pathologies.

Methods: A total of 83 gastritis patients confirmed by gastroscopy were enrolled, with 78 undergoing concurrent colonoscopies. The control group included 99 healthy subjects. Enzyme-linked immunosorbent assay was used to detect PGI, PGII, G17, and PGR. The performance of serological analysis for detecting gastritis pathology was evaluated using receiver operating characteristic (ROC) curves.

Results: The G17 and PGII levels increased significantly ($P < .001$), whereas PGR levels decreased ($P = .001$) in the gastritis group. The ROC analysis revealed that PGR had a sensitivity and specificity of 70.83% and 86.67%, respectively, in predicting *Helicobacter pylori*-infected gastritis and a sensitivity and specificity of 88% and 65.52%, respectively, in predicting active gastritis. The G17 levels were significantly elevated in gastritis patients undergoing concurrent colonoscopies ($P < .05$).

Conclusion: Pepsinogen I:pepsinogen II ratio was found to be a useful predictor of active gastritis and *H pylori*-infected gastritis. Furthermore, G17 was found to be closely related to pathological conditions found by colonoscopy and may provide recommendations for whether gastritis patients should undergo a concurrent colonoscopy.

Introduction

Gastritis is one of the most prevalent, severe, chronic, and insidious diseases in humans. More than half of the world's population is thought to suffer from chronic gastritis.[1] Gastritis can be classified into different types based on pathological findings, including active, intestinal metaplasia, atrophic, and *Helicobacter pylori* infection. Different types of gastritis will lead to different outcomes. Gastric cancer develops as result of host genetic and environmental interactions, and its progression is a multi-step process of gastritis-atrophy-metaplasia-dysplasia-cancer.[2] Therefore, atrophic gastritis and intestinal metaplasia are generally considered to be precancerous gastric lesions. *Helicobacter pylori* infection is considered to be an important initiating and promoting step in this cancer cascade.[3] Although nearly all subjects with *H pylori* infection have gastritis, only about 10% of those infected with chronic active gastritis progress to severe atrophic gastritis.[3] Moreover, in long-term follow-up studies, the risk of progression to gastric cancer was 0.1% for atrophic gastritis/intestinal metaplasia and 6% for atypical hyperplasia.[4] Therefore, it is important for clinical testing to identify the different types of gastritis.

The noninvasive serum markers pepsinogen I (PGI), pepsinogen II (PGII), gastrin-17 (G17), and pepsinogen I:pepsinogen II ratio (PGR) have recently been proposed as a new tool for predicting various gastric pathologies.[5-10] Pepsinogen is the precursor of pepsin and is divided into 2 subtypes, PGI and PGII.[11] Pepsinogen I is secreted mostly by the main cells in the fundus of the stomach, whereas PGII is secreted by cells throughout the stomach and duodenum.[12] Pepsinogen I can effectively reflect the function of gastric mucosa in acid secretion. The amount of gastric acid secreted is positively correlated with the level of PGI, so the reduction of gastric glands will lead to a decrease in serum PGI levels. Pepsinogen II levels were relatively constant. Gastrin-17 is a gastrointestinal hormone secreted by G cells in the stomach, which could effectively reflect the secretion function of gastric antrum and is closely related to the degree of atrophy of gastric antrum mucosa.[13] Therefore, PGI, PGII, and G17 are closely related to different pathological states of gastritis.

At the same time, gastroscopy is required when the patient is in a high-risk group for gastritis. In addition, some patients require a gastroscopy in addition to a colonoscopy to ensure a thorough examination of the digestive tract. Thus, it is worth considering whether there are serological indicators that can help clinicians determine whether colonoscopy should be performed alongside gastroscopy in patients with gastritis.

Our study aimed to investigate the relationship between serological indicators and gastritis as well as its association with various pathologies and whether gastritis patients require a colonoscopy.

Methods

Population Recruitment for the Study

From January 2021 to June 2021, 83 gastritis patients diagnosed by gastroscopy and histopathological analysis were enrolled at Wenzhou People's Hospital. These patients had not received systematic acid suppression prior to endoscopy. Exclusion criteria included: (1) patients with immune system diseases or other malignant tumors, (2) patients with a history of gastrointestinal surgery, and (3) pregnant and lactating women. The gastritis group included 45 men and 38 women, with a mean age of 54.12 ± 9.71 years. The control group included 99 healthy subjects who underwent physical examinations at the same time, including 57 men and 42 women, with a mean age of 55.29 ± 10.41 years. This study was approved by the Medical Ethics Committee of the Wenzhou People's Hospital (KY-2022-272). All participants signed informed consent.

Endoscopy and Histopathological Evaluation

All subjects underwent gastroscopy and histopathological analysis. During gastroscopy, 1 or more biopsy specimens were obtained from the lesion area. The lesion area was sampled by professional endoscopists, and the sampling sites were erosions first, followed by erythema. If no erosions were found, the sample was taken from erythema. Experienced pathologists graded biopsy specimens for chronic inflammation, activity, intestinal metaplasia, atrophy, and *H pylori* infection. The operative link on gastritis assessment (OLGA) staging system and operative link for gastritis intestinal metaplasia assessment (OLGIM) staging system were used to assess the degree of atrophy and intestinal metaplasia, respectively. A total of 78 patients in the gastritis group underwent colonoscopy at the same time. Pathological findings from colonoscopy were also collected and analyzed.

Blood Samples

Fasting blood samples from the 2 groups were collected for the detection of serum G17, PGI, PGII, and anti-*H pylori* antibodies. After the blood was collected, it was centrifuged at 1500*g* for 10 minutes to obtain serum, which was temporarily stored at 4°C. Serum detection was completed in 2 hours.

Serological Measurements

Serum PGI, PGII, and G17 levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (Biohit Biotech; catalog Nos. 601010.01CN, 601020.02CN, and 601035CN), and the procedure was carried out per the manufacturer's instructions. Briefly, serum and all calibrators were added to microplates coated with antibodies against PGI, PGII, and G17. After 1 hour of incubation at room temperature, the microplates were washed 3 times to remove residual samples. Horseradish peroxidase-labeled monoclonal antibodies were added to the microplates and incubated for another hour at room temperature. After incubation, the microplates were washed again, and the tetramethylbenzidine substrate solution was added. After 30 minutes at room temperature in the dark, the reaction was terminated by adding the stop solution, and the absorbance of the sample was measured at 450 nm. The PGR value was calculated using the following formula: PGR = PGI/PGII. The SD Bioline *H Pylori* kit (Standard Diagnostics) was used to detect serum anti-*H pylori* immunoglobulin (Ig)G, IgA, and IgM antibodies using a colloidal gold method.

Statistical Analyses

Wilcoxon rank sum tests were used to compare serum G17, PGI, PGII, and PGR levels in 2 groups. The same statistical methods were used to compare the 4 serological levels and clinicopathological findings in the gastritis group. A *P* value less than .05 was considered statistically significant. The performance of serological analysis for detecting the pathology of gastritis was evaluated using receiver operating characteristic (ROC) curves. Statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) version 22.0.

Results

G17, PGII, and PGR Levels Differed Significantly Between Gastritis and Control Groups

TABLE 1 shows the comparison of the 4 serological levels in gastritis and control groups. The G17 and PGII levels increased significantly in the gastritis group (median: 5.3 pmol/L, 12.91 μg/L) compared with the control group ($P < .001$). The PGR value in patients with gastritis was significantly lower than in healthy subjects ($P = .001$). However, no significant difference in PGI was found between the 2 groups ($P > .05$). At the same time, we excluded the *H pylori*–negative gastritis and compared the serological levels of the *H pylori*–positive gastritis and the control group. There were significant differences in G17, PGII, and PGR (Table S1). In addition, we classified *H pylori*–negative gastritis into the control group and compared their serological levels with those of *H pylori*–positive

TABLE 1. Serological levels between the gastritis group and control group

	n	G17		PGI		PGII		PGR	
		Median (pmol/L)	*P*	Median (μg/L)	*P*	Median (μg/L)	*P*	Median	*P*
Control group	99	1.8	<.001	97.59	.078	7.92	<.001	12.21	.001
Gastritis group	83	5.3		108.72		12.91		8.85	

G17, gastrin-17; PGI, pepsinogen I; PGII, pepsinogen II; PGR, pepsinogen I:pepsinogen II ratio

gastritis. The same difference was found in G17, PGII, and PGR (Table S2).

G17 Had a Higher Sensitivity in Predicting Gastritis

FIGURE 1 shows the ROC curves for G17, PGI, PGII, and PGR in predicting gastritis. The area under the ROC curves (AUROC) for G17, PGI, PGII, and PGR were 0.727 (95% CI, 0.652-0.802), 0.576 (95% CI, 0.490-0.662), 0.654 (95% CI, 0.571-0.736), and 0.643 (95% CI, 0.561-0.725), respectively. The sensitivities of G17, PGI, PGII, and PGR were 75.76% (cut-off value, 3.87 pmol/L), 61.62% (cut-off value, 1.31 μg/L), 71.72% (cut-off value, 1.8 μg/L), and 72.73% (cut-off value, 1.72), respectively. The specificities of G17, PGI, PGII, and PGR were 62.65%, 53.01%, 60.24%, and 57.83%, respectively. Gastrin 17 had a higher sensitivity and specificity for predicting gastritis.

PGR Could Predict Active Gastritis and *H pylori*–Infected Gastritis

Gastroscopy revealed that 54 patients in the gastritis group had only a single gastritis lesion site. The majority of the single lesion sites (n = 50) were found in the gastric antrum. Based on the gastroscopy pathological findings, chronic inflammation, activity, intestinal metaplasia (using the OLGIM staging system), atrophy (using OLGA staging system), and *H pylori* infection degrees of the biopsy specimen were evaluated. As shown in **TABLE 2**, G17, PGI, PGII, and PGR did not differ in their chronic inflammation, whether assessed by OLGA or OLGIM.

The activity was divided into 3 groups: 0 (negative activity at the lesion site), ≥1+ (positive activity at the lesion site), and not performed. The PGR value was significantly lower in patients with active gastritis ($P < .001$, **TABLE 2**), whereas there was no difference between active and inactive gastritis in terms of PGI and G17 levels. The ROC curves demonstrated the predictive ability of G17, PGI, PGII, and PGR for active gastritis. For patients with active gastritis, the AUROC values for G17, PGI, PGII, and PGR were 0.625 (95% CI, 0.475-0.774), 0.536 (95% CI, 0.379-0.693), 0.657 (95% CI, 0.504-0.809), and 0.804 (95% CI, 0.684-0.925), respectively (**FIGURE 2A**). Clearly, the AUROC of PGR was the largest of the 4 serological markers, and 11.22 was the best cut-off value for PGR (sensitivity 88%, specificity 65.52%; **FIGURE 2A**).

The *H pylori* infection degree of the biopsy specimen was divided into 2 groups: 0 (the *H pylori* infection of the lesion site is negative)

FIGURE 1. ROC curves of G17 (AUROC = 0.727) (A), PGI (AUROC = 0.576) (B), PGII (AUROC = 0.654) (C), and PGR (AUROC = 0.643) (D) for the diagnosis of gastritis. AUROC, area under the receiver operating characteristic curve; G17, gastrin-17; PGI, pepsinogen I; PGII, pepsinogen II; PGR, pepsinogen I:pepsinogen II ratio; ROC, receiver operating characteristic.

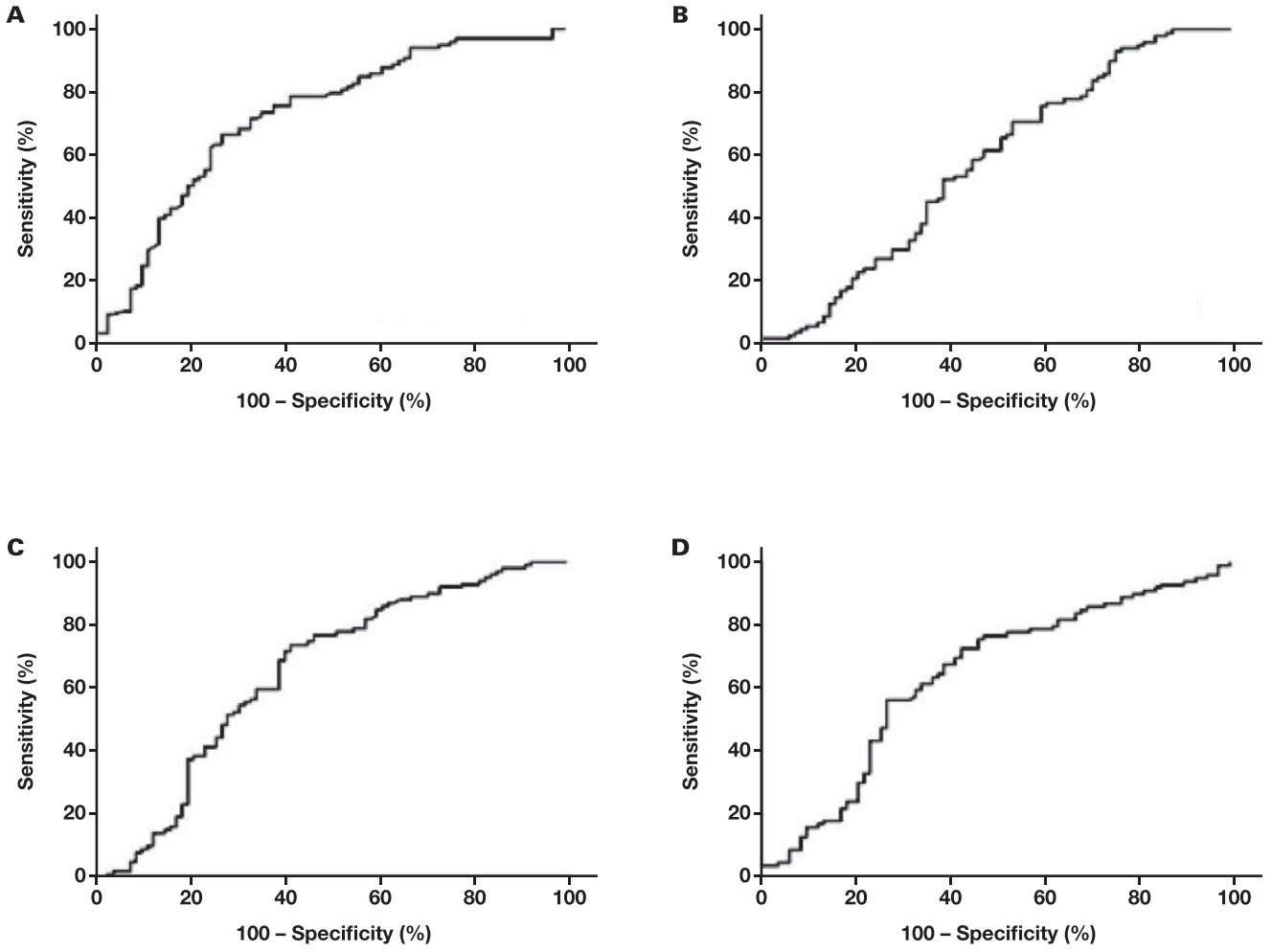

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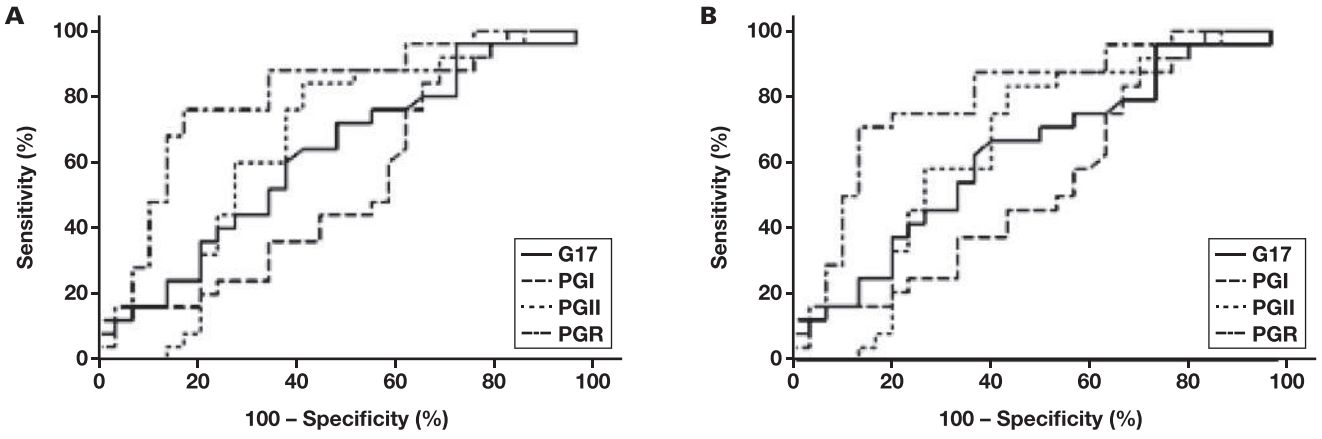
TABLE 2. Serological levels in patients with single gastritis site (n = 54)

	n	G17		PG I		PG II		PGR	
		Median (pmol/L)	*P*	Median (μg/L)	*P*	Median (μg/L)	*P*	Median	*P*
Single gastritis site									
Antrum	50	5.40	.893	99.14	.347	11.55	.562	9.79	.21
Gastric angle	2	5.48		195.65		13.70		16.99	
Gastric body	1	3.93		117.54		23.52		5.00	
Pylorus	1	2.85		179.55		22.38		8.02	
Chronic inflammation									
1+	11	6.22	.925	104.31	.682	8.10	.861	9.74	.898
>1+	39	3.95		115.36		13.59		9.99	
NA	4	—		—		—		—	
Activity									
0	29	3.91	.133	99.46	.809	8.04	.049	12.35	<.001
≥1+	24	5.97		114.11		16.49		6.92	
NA	1	—		—		—		—	
OLGIM									
0	36	5.40	.317	114.19	.107	12.49	.542	9.92	.219
≥I	17	3.93		98.28		11.48		8.02	
NA	1	—		—		—		—	
OLGA									
0	45	4.29	.318	111.58	.756	12.43	.535	9.85	.121
≥I	4	8.62		100.38		17.08		6.51	
NA	5	—		—		—		—	
H pylori infection degree of biopsy specimen									
0	30	3.93	.012	101.89	.595	8.07	.058	12.01	<.001
≥1+	24	6.36		109.38		16.49		6.75	

G17, gastrin-17; NA, not performed; OLGA, operative link on gastritis assessment; OLGIM, operative link for gastritis intestinal metaplasia assessment; PGI, pepsinogen I; PGII, pepsinogen II; PGR: pepsinogen I:pepsinogen II ratio; —, due to the limited sample size, this group was not suitable for statistical comparison.

FIGURE 2. ROC curves for G17, PGI, PGII and PGR in predicting active gastritis (A) and *H pylori*–infected gastritis (B). G17, gastrin-17; PGI, pepsinogen I; PGII, pepsinogen II; PGR, pepsinogen I:pepsinogen II ratio; ROC, receiver operating characteristic.



and ≥1+ (the *H pylori* infection of the lesion site is positive). Pepsinogen I:pepsinogen II ratio decreased significantly in *H pylori*–infected gastritis ($P < .001$), whereas G17 increased ($P = .012$, **TABLE 2**). In **FIGURE 2B**, ROC curves for predicting *H pylori*–infected gastritis are shown based on G17, PGI, PGII, and PGR. The AUROC values for G17, PGI, PGII, and PGR were 0.631 (95% CI, 0.481-0.780), 0.542 (95%

CI, 0.387-0.699), 0.651 (95% CI, 0.501-0.802), and 0.800 (95% CI, 0.679-0.922), respectively. According to the ROC graph, PGR had a sensitivity and specificity of 70.83% and 86.67%, respectively, with a cut-off value of 8.045 for predicting *H pylori*–infected gastritis (**FIGURE 2B**).

G17 Levels Were Significantly Correlated With the Colonoscopy Outcomes of Gastritis Patients

In the gastritis group, the serological levels of G17, PGI, and PGII were compared with clinical characteristics such as sex, age, colonoscopy, gastritis site, and serum anti-*H pylori* antibodies. Significant differences in G17, PGI, and PGII levels were found in the gastritis group among different age groups, with a tendency to increase further as age progressed (**TABLE 3**) (P_{G17} = 0.016, P_{PGI} = 0.003, P_{PGII} = 0.004). PGR decreased more in gastritis patients whose serum contained anti-*H pylori* antibodies ($P < .05$).

In the gastritis group, 78 patients underwent colonoscopy in addition to gastroscopy. Therefore, we divided the gastritis patients into 2 groups based on the pathological findings of the colonoscopy: a group of 31 patients with no obvious colorectal abnormalities and a group of 47 patients with colorectal changes. Polyps were found in 42 patients with colorectal changes, with the remaining 5 patients having colonic lipoma, multiple diverticula in the large intestine, proctitis, and ileocecal valve ulceration. Only G17 levels were significantly elevated ($P < .05$) among the 4 serological indicators in patients with colorectal changes (**TABLE 3**). The ROC curves for predicting colorectal changes based on G17, PGI, PGII, and PGR are shown in **FIGURE 3**. The AUROC values for G17, PGI, PGII, and PGR were 0.662 (95% CI, 0.539-0.785), 0.611 (95% CI, 0.486-0.736), 0.588 (95% CI, 0.459-0.716), and 0.527 (95% CI, 0.398-0.655), respectively. The sensitivity and specificity of G17, PGI, PGII, and PGR were 70.21%, 61.70%, 68.09%, and 61.70%, and 61.29%, 51.61%, 51.61%, and 51.61%, respectively.

Discussion

Gastritis remains one of the most prevalent pandemic infections, with serious consequences such as peptic ulcers and stomach cancer.[14] Therefore, diagnosing gastritis and analyzing its pathology is critical. In comparison with gastroscopy, a serological examination is noninvasive and highly acceptable. Therefore, investigating the relationship between serological indices and the clinicopathology of gastritis is a new direction for the noninvasive diagnosis of gastritis.

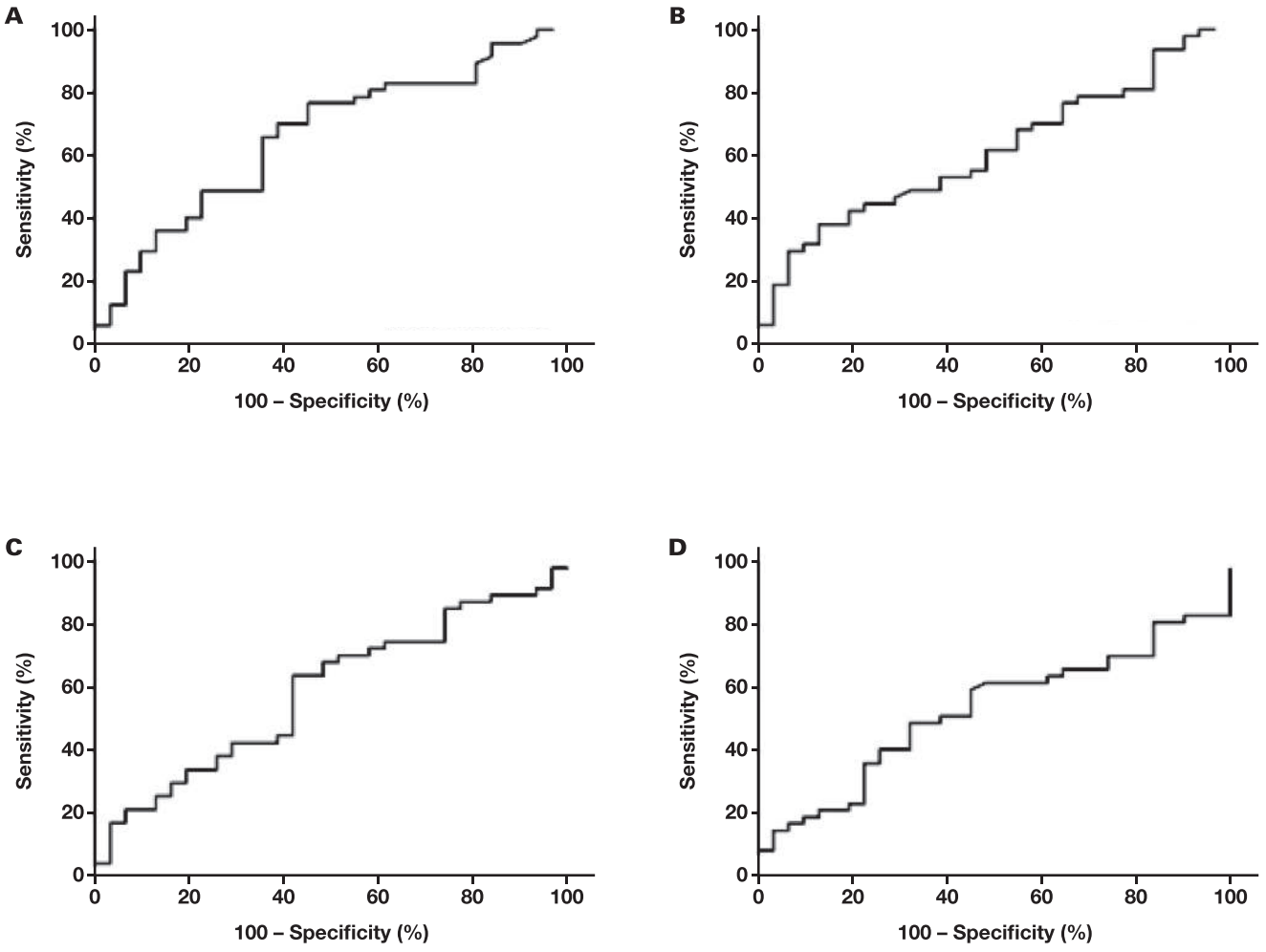
In this study, we selected 4 serological indicators of gastric function: G17, PGI, PGII, and PGR. We found that G17, PGII, and PGR differed significantly between the gastritis and control groups and that G17 and PGII were significantly increased in the gastritis group, which was consistent with previous studies.[15] Pepsinogen I and PGII are pepsins derived from different areas of the stomach and duodenum, respectively.[16] Pepsinogen I is secreted by the chief cells of the gastric body and fundus, whereas gastric acid is secreted by parietal cells. Some studies have shown that PGI levels are positively correlated with maximum gastric acid secretion. Pepsinogen II is produced by mucosal cells in the pylorus.[5,17-19] Gastrin is a gastrointestinal hormone secreted by G cells in the gastric antrum and duodenum that plays an important role in regulating the functions of the digestive tract and maintaining its structural integrity. Gastrin 17 is the most abundant and important subtype of gastrin in humans. It is only secreted by G cells in the antrum.[20] Serum PG and G17 have been shown to have good sensitivity and specificity in detecting early-stage cancer.[5,17,20-23] According to our study, G17

TABLE 3. Serological levels under different clinical characteristics in the gastritis group (n = 83)

	n	G17		PG I		PG II		PGR	
		Median (pmol/L)	*P*	Median (μg/L)	*P*	Median (μg/L)	*P*	Median	*P*
Sex									
Male	45	6.93	.358	118.65	.354	12.42	.602	9.60	.098
Female	38	4.16		104.47		14.26		8.05	
Age, y									
≤45	16	2.17	.016	77.50	.003	7.18	.004	10.26	.282
46-74	65	5.50		117.54		13.59		8.85	
≥75	2	19.63		230.99		32.64		7.40	
Colonoscopy and histological findings									
No colorectal abnormalities	31	3.23	.016	99.46	.098	8.97	.193	8.50	.69
Colorectal changes	47	5.65		112.85		13.17		9.71	
Not performed	5	—		—		—		—	
Gastritis sites									
One site	54	5.30	1	105.11	.445	12.03	.406	9.79	.16
Multiple sites	29	5.27		124.63		13.78		8.25	
Serum anti-*H pylori* antibody									
Negative	42	4.12	.243	107.56	.537	12.02	.206	9.86	.026
Positive	20	5.27		93.06		15.37		7.18	
Not performed	21	—		—		—		—	

G17, gastrin-17; PGI, pepsinogen I; PGII, pepsinogen II; PGR, pepsinogen I:pepsinogen II ratio.

FIGURE 3. ROC curves of G17 (AUROC = 0.662) (A), PGI (AUROC = 0.611) (B), PGII (AUROC = 0.588) (C), and PGR (AUROC = 0.527) (D) for the diagnosis of colorectal changes in gastritis patients. AUROC, area under the receiver operating characteristic curve; G17, gastrin-17; PGI, pepsinogen I; PGII, pepsinogen II; PGR, pepsinogen I:pepsinogen II ratio; ROC, receiver operating characteristic.



had a sensitivity and specificity of 75.76% and 62.65%, respectively, for predicting gastritis. Gastroscopy combined with biopsy histopathology is the gold standard for determining the status of gastric and duodenal mucosa.[24] However, due to the unpleasant procedure and inconvenience of intestinal preparation before examination, this examination is quite difficult to accept for most patients. Another difficulty in early endoscopic screening in China is the lack of essential equipment and professional endoscopists at grassroots-level hospitals. Additionally, due to the invasive nature of endoscopy, there were risks of coughing, gastrointestinal mucosal injury, and bleeding during the operation. Thus, early endoscopic screening in China is not implemented in large-scale health examinations, and this results in many patients being diagnosed with advanced-stage tumors on initial discovery. The use of G17 as a serological indicator is more noninvasive and has no radiation risk, which was easily accepted by patients.

In terms of clinical characteristics of gastritis patients, values of G17, PGI, and PGII were significantly related to age and tended to increase further as age progressed. This could be due to degenerative changes in the gastric mucosa, insufficient blood supply, poor secretion function, mucosal barrier dysfunction, and other factors associated with aging.[25] However, pepsinogen and gastrin secretion did not differ between men and women, although men had slightly higher levels than women.

Among the enrolled gastritis patients, G17 levels were significantly lower in patients with no obvious intestinal mucosa abnormalities than in the other group. Recent study elucidated that G17 and N-carboxymethyl gastrin stimulate the growth of colon cancer cells both in vivo and in vitro.[26] In our study, G17 had a sensitivity and specificity of 70.21% and 61.92%, respectively, for predicting colorectal changes in patients with gastritis. In addition, G17 can help clinicians determine whether gastritis patients should undergo gastroscopy and colonoscopy at the same time. However, a larger sample size is required to define the G17 threshold for predicting colonic pathological changes.

There are currently some studies on the use of serum pepsinogen to predict atrophic gastritis and *H pylori* chronic gastritis.[27-29] However, the prediction of active gastritis is rare. Pei et al[13] developed a logistic regression model based on *H pylori* infection; using PGI, G17, and the number of lesions to predict intestinal metaplasia in atrophic gastritis, with an AUROC of 0.859. However, no significant correlation was found

between PGI and G17 and gastritis intestinal metaplasia in this study, which could be attributed to the small number of patients in the study, with 66.7% of the patients not having intestinal metaplasia. Kitamura et al[30] enrolled 4483 gastritis patients in Japan and found that the PGR value of the gastritis patients with *H pylori* infection was significantly lower than that of the negative patients, and the sensitivity and specificity of the gastritis diagnosis of *H pylori* infection were both greater than 90% when the PGR threshold was 5. In our study, PGR had the sensitivity and specificity of 70.83% and 86.67%, respectively (cut-off value: 8.045), in predicting gastric mucosal *H pylori* infection. It is possible that differences in ethnicity, environment, and diet result in different thresholds. Takeichi et al[31] found that gastric cancer progresses as gastritis-atrophy-metaplasia-cancer and partly on direct carcinogenesis based on active inflammation. Meanwhile, highly active inflammation in the nonatrophic gastric body leads to the formation of gastric folds.[32] Therefore, it is critical to predict the onset of active gastritis and to intervene early for clinical treatment. In our study, PGR was significantly lower in active gastritis than in inactive gastritis and was predictive of active gastritis with sensitivity and selectivity of 88% and 65.52%, respectively.

There were some limitations to this study that need careful consideration. First, this was a hospital-based cross-sectional population, not a natural population. There may be some sampling bias. Second, gastroscopic lesions were taken from erythema or erosions by endoscopists, although erythema was not a good predictor of histological outcome. Upper gastrointestinal endoscopy is not mandatory in large-scale health examinations in China, which limited the elimination of bias in a cross-sectional study.

In conclusion, serum biomarkers were found to be a useful indicator for predicting the pathological state of the stomach, particularly PGR, which could reflect active gastritis and *H pylori* infective gastritis. Furthermore, G17 could provide recommendations with the results of colonoscopy pathology in gastritis patients. However, although PGR and G17 levels may be a noninvasive measure for predicting gastritis, the decision-making based on PGR and G17 must be further validated in prospective epidemiological studies.

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Conflict of Interest Disclosure

The authors have nothing to disclose.

Data Availability

Data supporting this research article are available from the corresponding author on reasonable request.

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CASE STUDY

Familial nonmedullary thyroid cancer: a case series in Iranian patients with a meta-review of case series

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Key words: familial; nonmedullary; thyroid; cancer; whole-exome sequencing; *SRGAP1; FOXE1*

Abbreviations: NMTC, nonmedullary thyroid cancer; FNMTC, familial NMTC; WES, whole-exome sequencing; PTC, papillary thyroid cancer; BWA, Burrows-Wheeler Aligner; GATK, Genome Analysis Toolkit; SNP, single nucleotide polymorphism; CNV, copy number variants; VCF, Variant Call Format; EXAC, Exome Aggregation Consortium; GnomAD, Genome Aggregation Database; ACMG, American College of Medical Genetics and Genomics; *SRGAP1*, Slit-robo rho GTPase-activation protein 1; *FOXE1*, Forkhead boxE1; NGS, next-generation sequencing; Cdc42, cell division cycle 42; GWAS, genome-wide association study

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ABSTRACT

Background: Nonmedullary thyroid cancer (NMTC) comprises approximately 90% of all thyroid cancers, and about 3% to 9% of NMTC cases have a familial origin. Familial NMTC (FNMTC) in the absence of a documented familial cancer syndrome such as Cowden syndrome is characterized by the occurrence of thyroid cancer of follicular cell origin in 2 or more first-degree relatives.

Methods: Whole-exome sequencing (WES) was used to identify pathogenic genetic variants in 2 Persian families with FNMTC. The purpose of this work is to assess the pathogenic status of these variants as well as the cosegregation status of the variants observed in the examined families.

Results: By analyzing WES data in the first family, *SRGAP1*: NM_020762: exon16: c.C1849T was identified as a pathogenic variant. This variant was confirmed by Sanger sequencing. In the second family, the variant *FOXE1*: NM_004473: exon1: c.531_532insCGCGA was identified but was not confirmed by Sanger sequencing.

Conclusion: Based on the data, *SRGAP1* can be a potential candidate gene for susceptibility to FNMTC in the first family. However, additional analyses like whole genome sequencing and copy number variations are required to ascertain the disease status in second family.

Introduction

Thyroid cancer is a common endocrine malignancy whose incidence is increasing worldwide.[1] Although this increasing incidence can be the result of improvements and progress in screening and diagnosis methods, this progress does not justify the diagnosis of symptomatic large tumors.[2,3] It is the fifth most prevalent cancer in women, with a 3:1 female-to-male ratio.[4,5] Studies in Southwest Asian nations indicate that the prevalence of thyroid cancer ranges from 1.1% to 3.5% in men and from 2.6% to 12.1% in women. Iran appears to be situated in the middle of the locations with the largest and smallest prevalence, according to one study's incidence rate of 3.5%.[6] With a median age of 51 years at diagnosis, thyroid cancer affects a younger population than most other cancers. Known risk factors for thyroid cancer include excessive exposure to ionizing radiation especially during childhood, gender, and positive family history.[7] Thyroid cancer is classified into 2 main types: medullary thyroid cancer, which originates from parafollicular C cells, and nonmedullary thyroid cancer, which originates from follicular epithelial cells.[8] About 90% of thyroid cancers are sporadic, and 3% to 9% of these cancers are familial.[9] Additionally, cancer syndromes that run in families are responsible for 5% of familial nonmedullary thyroid cancer (FNMTC). These include Cowden syndrome, familial adenomatous polyposis, Gardner syndrome, Carney complex, Werner syndrome, and DICER1 syndrome.[10] Although the genetic changes that cause this cancer have been found in syndromic cases, there is not enough proof for the underlying genetic changes, and the main sign of the disease in the nonsyndromic form of thyroid cancer is that it spreads to the thyroid gland. Thyroid cancer, compared with other cancers such as colon, breast, and prostate, shows the highest relative familial risk. Studies have shown that the risk of developing nonmedullary thyroid cancer (NMTC) in first-degree relatives is 8 to 10 times higher than in the general population.[11-14] Therefore, a positive family history is a critical finding for FNMTC diagnosis. So, FNMTC is a well-differentiated thyroid cancer that starts in follicular cells in 2 or more first-degree relatives when there are no environmental factors that cause them to be more likely to get it.[15,16] Several researchers reported that FNMTC is more likely to form bilateral, multifocal tumors that can invade lymph nodes and increase the chance of recurrence.[17] FNMTC is a rare cancer syndrome that most likely presents as an autosomal dominant pattern with

incomplete penetrance and variable expression. People are more likely to get this cancer because of several genes (*SRGAP1, NKX2-1*, *HABP2*, and *FOXE1*) and chromosomal loci (1q21, 6q22, 8p23, 1p22, 2q21, 14q32, and 8q24). This shows that thyroid cancer has heterogeneous origins.[12,13,18,19] Meanwhile, genetic testing is still not advised for diagnosis, treatment, or follow-up decisions because many of these loci or susceptibility genes have not been further validated in subsequent research. It is, however, crucial to identify predisposing genes or susceptibility loci as a tool for screening at-risk people and to support genetic counseling.[8]

Because there are not enough data about the genetic causes of FNMTC cases in the Iranian population, the current study was designed to identify the pathogenic genetic variants in 2 Persian families affected by FNMTC.

Methods

This is a case series of molecular studies in which, using high-throughput genomics and bioinformatics tools, potential pathogenic genetic variants have been evaluated and reported.

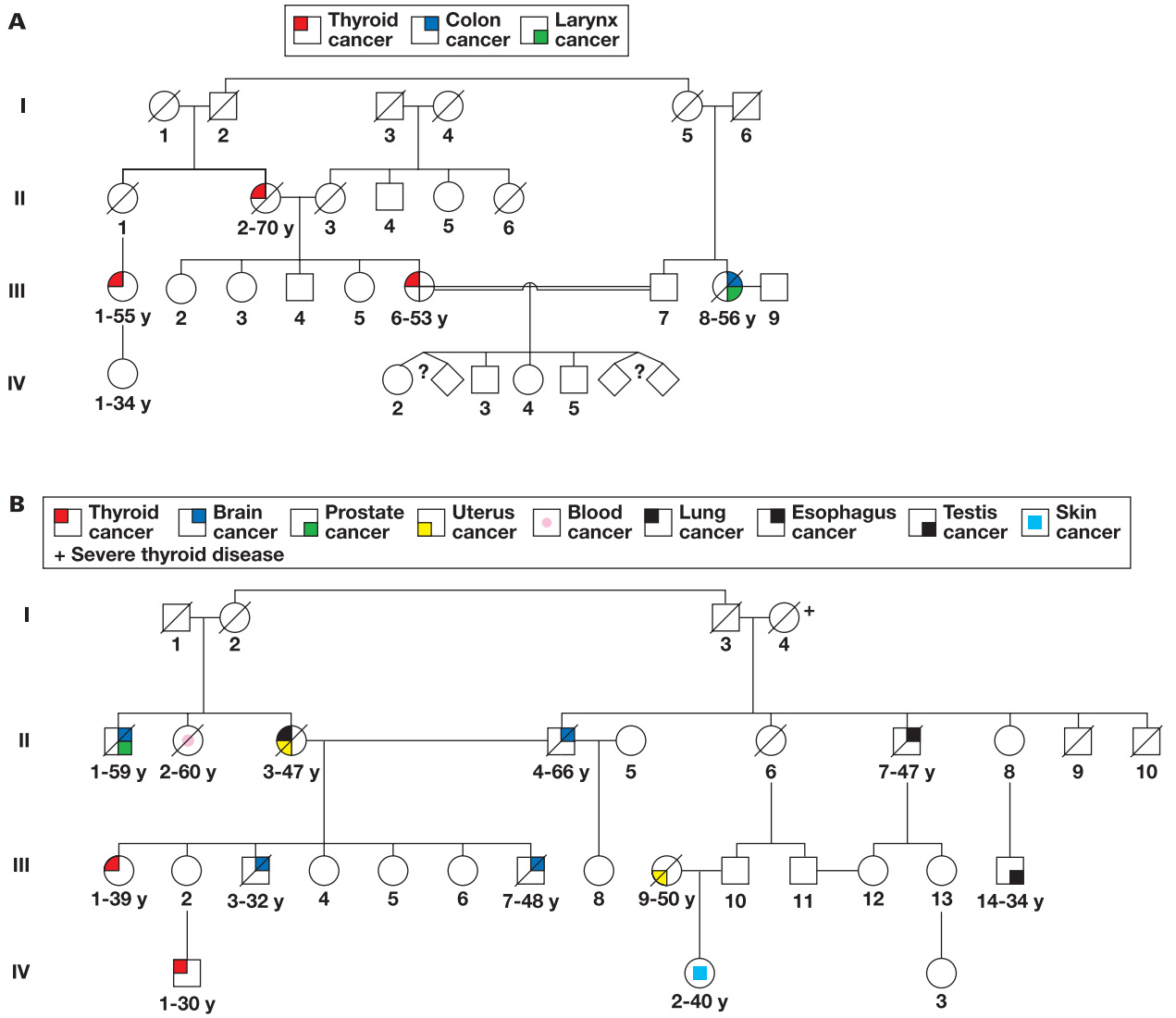
Case Selection and DNA Isolation

The study included 2 Persian families affected by FNMTC who registered with MACSA, a referral charity-based institute for cancer prevention and control in central Iran. All clinical and diagnostic documents of the patients registered in MACSA are routinely recorded in an electronic database. The criteria for choosing families for this study are the presence of at least 2 thyroid cancer patients with an age of onset lower than 50 years in 2 consecutive generations and the availability of at least 1 patient's blood or healthy tissue sample. Additionally, no known hereditary cancer disorders were identified in the families that were studied.

In family 1, the proband was a 58-year-old woman (III-6) with papillary thyroid cancer (PTC). She had undergone a thyroidectomy several years ago. Rather than PTC, there were cases of colon and larynx cancers in this family (**FIGURE 1A**).

In family 2, the proband was a 43-year-old woman (III-1) with PTC who had undergone a thyroidectomy the previous year. There was a history of several malignancies, including brain, blood, lung, uterine, prostate, esophagus, breast, skin, and testicles among the members of this family within at least 3 successive generations (**FIGURE 1B**).

FIGURE 1. Pedigree charts for family 1 (A) and family 2 (B). Squares represent males, circles represent females, slashes indicate deceased members, and y shows age. The star indicates the whole-exome sequencing.



After genetic counseling and informed consent, peripheral blood samples were collected from probands and other affected and healthy members of the families. Genomic DNA was extracted by following the instructions on a DNA extraction kit (Qiagen). A nanodrop (a type of spectrophotometer) and gel electrophoresis were used to check the amount and quality of the DNA that was extracted. The workflow in this study can be seen in the summary (**FIGURE 2**).

Whole-Exome Sequencing and Variant Screening to Find Possible Genes That Cause Nonsyndromic FNMTC

The whole-exome sequencing (WES) technique evaluated the genomic DNA of both probands. The sequencing was performed using Sure Select Human All Exon V7 (Agilent Technologies) by Novogene and sequenced on an Illumina HiSeq 4000 with coverage of 100x. (Illumine). After sequencing, the data were processed in the 3 main stages of mapping, variant calling, and annotation. Mapping was done using the Burrows-Wheeler Aligner (BWA) tool, variant calling with the Genome Analysis Toolkit (GATK) tool, and annotation by Annovar. Our reasons for choosing these tools were that the BWA/GATK software package is more sensitive than some software, and the output of BWA is used with minimal processing by the tool software packages (Picard and SAMtools) as the input of GATK tools. BWA, GATK, and Annovar are available and free of charge.[20] Annovar is also an efficient, adaptable, extensible, and cross-platform tool for annotating genetic variations from different genomes using the latest data. In addition, it can use different types of input files and can annotate for single nucleotide polymorphisms (SNPs), copy number variants (CNVs), and INDEL variants.[21] The steps of WES and variant screening are summarized in (**FIGURE 3**).

Then, the data from the Variant Call Format (VCF) was filtered based on how often it appeared in population databases like the 1000 Genome Project, the Exome Aggregation Consortium (EXAC), and the Genome Aggregation Database (GnomAD) with a frequency of less than 0.01. Moreover, gene panels exposing genes connected to this cancer were created along with a list of suggested variants indicated in prior studies. Then, the shared variants between the made gene list and the sequencing-based VCF were found. The variants produced by this sharing were assessed using both the American College of Medical Genetics and Genomics (ACMG) guidelines and bioinformatics methods, with the results displayed in **TABLE 1** for family 1 and **TABLE 2** for family 2.

To make the table of characteristics of the variants shorter, "benign" and "likely benign" variants were left out. In **TABLE 2**, only variants that the ACMG guidelines called "uncertain significance," "likely pathogenic," or "pathogenic" are shown.

Sanger Sequencing and Cosegregation Analysis

Sanger sequencing was performed to confirm the candidate variants and co-segregation analysis of the candidate variants in the families. For this purpose, a pair of primers were designed for each family using http://primer3.ut.ee as follows: primer for family 1

FIGURE 2. Workflow in this study.

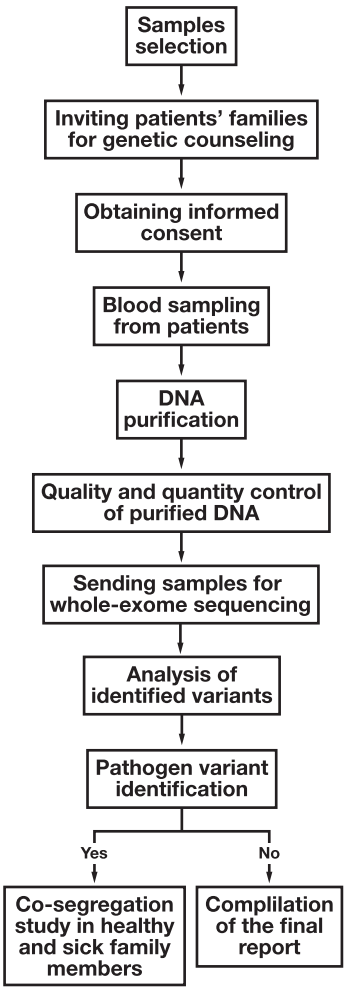


FIGURE 3. The steps of whole-exome sequencing and variant screening.

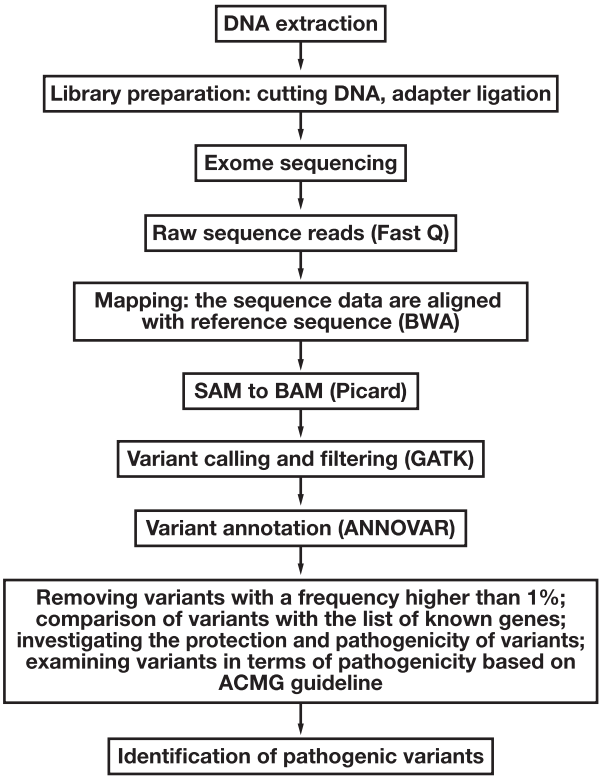


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TABLE 1. Candidate susceptibility variants identified in family 1[a]

Gene	Mutation position	Accession number	Nucleotide change	Protein effects	Mutation classification	dbSNP	SIFT	PolyPhen-2	ACMG guideline interpretation
PCNX2	Chr1: 2.33E+08	NM_014801	C>T	p.A1989T	Nonsynonymous SNV	rs189263803	Tolerated (1)	Benign (0)	Likely benign
ACKR3	Chr2: 2.37E+08	NM_020311	C>T	p.H298Y	Nonsynonymous SNV	rs150632398	Deleterious (0.011)	Damaging (1)	Likely benign
DKK1	Chr10: 54074798	NM_012242	G>T	p.R120L	Nonsynonymous SNV	rs149268042	Deleterious (0)	Damaging (1)	Likely benign
WNK1	Chr10: 54074798	NM_012242	CAACTAGTTdel	p.T1600_S1602del	Non-frameshift deletion	rs544395150			Benign
UVRAG	Chr11: 75851814	NM_003369	G>T	p.G486V	Nonsynonymous SNV	rs77892162	Tolerated (0.224)	Benign (0.328)	Benign
GLI1	Chr12: 57858576	NM_001167609	C>A	p.S64Y	Nonsynonymous SNV	rs139570630	Deleterious (0)	Damaging (1)	Likely benign
EPCAM	Chr2: 47607081	NM_002354	A>G	p.I277M	Nonsynonymous SNV	rs115283528	Deleterious (0.017)	Probably damaging (0.61)	Likely benign
RASGRP3	Chr2: 33752240	NM_015376	G>A	p.G282S	Nonsynonymous SNV	rs142000180	Tolerated (0.443)	Damaging (0 977)	Benign
SRGAP1	Chr12: 64502747	NM_001346201	C>T	p.R594C	Nonsynonymous SNV	rs114817817	Deleterious (0.001)	Damaging (1)	Uncertain significance
GGT2	Chr22: 21579720	NM_001351304	C>T	p.A42T	Nonsynonymous SNV	rs543952156	Deleterious (0)		Likely benign
APOL1	Chr22: 36661354	NM_001136541	C>T	p.L140F	Nonsynonymous SNV	rs148296684	Deleterious (0.013)	Damaging (0.999)	Benign
EVPL	Chr17: 74006618	NM_001320747	G>A	p.L912F	Nonsynonymous SNV	rs141762313	Deleterious (0.044)	Probably damaging (0.778)	Likely benign
ST6GALNAC2	Chr17: 74564830	NM_006456	C>T	p.L274L	Nonsynonymous SNV	rs117281881			Benign

ACMG, American College of Medical Genetics and Genomics.
[a]The SIFT score might be between 0.0 (deleterious) and 1.0 (tolerated). Variants with a score between 0.0 and 0.05 are deleterious. PolyPhen-2 scores range from 0.0 (tolerated) to 1.0 (deleterious). Variants with scores between 0.85 and 1 are more confidently predicted to be deleterious.

(F: ACCCAAGCCGATCATTTATTC; R: CTGCTGATAAATGGAACCCAAC); primer for family 2 (F: CATCCGCCACAACCTCAC; R: CCGCCAGGGTTGTAGCAG).

Primer blast (https://www.ncbi.nlm.gov/tools/primer-blast) was used to confirm where the primers bind exactly, and BLAT (https://genome.ucsc.edu/cgi-bin/hgBlat) was used to look for SNPs in the designed primers. The length of the polymerase chain reaction product was measured and used for Sanger sequencing after being viewed on a 1.5% agarose gel.

Results

The obtained WES data of the probands' genomic DNA was analyzed to pinpoint the genetic variants responsible for the emergence of nonsyndromic FNMTC.

In the first family, one of the potential variants thought to increase the risk of the disease was the *SRGAP1*: NM 020762:c.C1849T. Sanger sequencing confirmed the candidate change and cosegregation study analysis showed the presence of the candidate change in another patient member of this family (III-1) and a carrier of this change (IV-5).

In the second family, *FOXE1*: NM_004473: exon1: c.531_532insCGCGA: p. A178Rfs*191 was considered as a germline pathogenic variant for FNMTC, but Sanger sequencing did not confirm this variant.

Discussion

In this study, 2 Persian affected pedigrees with FNMTC have been presented. As mentioned, the genomic DNA of the probands was investigated using WES to determine the pathogenic variants. After variant prioritization stages and the identification of pathogenic variants, the cosegregation pattern of these variants was evaluated in the affected and healthy members of these families.

ACMG Guidelines for Classification and Interpretation of Variants

With the advancement of next-generation sequencing (NGS) technology and the significant increase in variants identified in patients, ACMG updated its standards and guidelines in 2015 with the help of experts and the use of experimental data. They presented a method for classifying sequence changes and explaining terms. In this manual, several criteria and evidence (for example, population data, computational data, functional data, and segregation data) are used to classify variants in terms of pathogenicity importance into 5 groups: "pathogenic," "likely pathogenic," "uncertain significance, ""benign," and "likely benign" groups (**TABLE 3**).

According to strength, these criteria are placed in 4 groups: "very strong," "strong," "medium," and "supportive" (**TABLE 4**). Finally, this manual provides rules for combining evidence according to which each variant is placed in 1 of the above 5 groups in terms of pathogenicity.[22]

TABLE 2. Variants identified in the results of whole-exome sequencing in family 2

Gene	Mutation position	Accession number	Reference	Variant	Protein effect	Mutation classification	Zygosity	ACMG guideline interpretation
WNK1	Chr12: 908031	NM_014823	C	G	p.Y2028	Stop gain	Homozygous	Uncertain significance
FOXE1	Chr9: 97854445	NM_004473		CGCGA	p.A178Rfs*191	Frameshift insertion	Heterozygous	Likely pathogenic
TEK	Chr9: 27213582	NM_001290078	T	A	p.Y844X	Stop gain	Heterozygous	Likely pathogenic
PCM1	Chr8: 17938873	NM_001352647	A	T	p.N159I	Nonsynonymous (SNV)		Uncertain significance
PLXCN1	Chr12: 94149730	NM_005761	C	G	p.Y253X	Stop gain	Heterozygous	Likely pathogenic
TRIM33	Chr1: 1.14E+08	NM_033020	C	A	p.K1110N	Nonsynonymous (SNV)	Heterozygous	Uncertain significance
AHNAK2	Chr14: 1.05E+08	NM_001350929		CAAAACCCCA	p.A1242Gfs*7	Frameshift insertion	Heterozygous	Likely pathogenic
AHNAK2	Chr14: 1.05E+08	NM_001350929	G	T	p.S1085X	Stop gain	Heterozygous	Uncertain significance
PTEN	Chr10: 87864144:	NM_001304717	G	A	p.C65Y	Nonsynonymous (SNV)		Uncertain significance
MRGPRX4	Chr11: 18173504	NM_054032	T	A	p.L83X	Stop gain	Homozygous	Uncertain significance
USP22	Chr17: 21021210	NM_015276	G	T	p.Y107X	Stop gain	Homozygous	Likely pathogenic
NRG1	Chr8: 32595840	NM_001160002	G	C	p.R38P	Nonsynonymous (SNV)		Uncertain significance
SEC23B	Chr20: 18532728	NM_001172745	C	G	p.P433R	Nonsynonymous (SNV)		Uncertain significance
HEY1	Chr8: 79766263	NM_001282851	TGTGGCA		p.M1?	Start loss	Heterozygous	Uncertain significance
G6PD	ChrX: 1.55E+08	NM_000402	A	T	p.Y467X	Stop gain	Homozygous	Likely pathogenic
AKT1	Chr14: 1.05E+08	NM_001014431	C	G	p.E242D	Nonsynonymous (SNV)		Uncertain significance
RBMXL3	ChrX: 1.15E+08	NM_001145346		CTCGCCCAACGCCCACAGCG	p.R393Tfs*121	Frameshift insertion	Heterozygous	Uncertain significance
EML4	Chr2: 42282878	NM_001145076	A	T	p.K225X	Stop gain	Homozygous	Pathogenic
PCNX2	Chr1: 233262967	NM_014801	C	G	p.R117T	Nonsynonymous (SNV)		Uncertain significance
MSH3	Chr5: 80654924	NM_002439		CGCCTTCCCGC	p.P67Afs*17	Frameshift insertion	Heterozygous	Likely pathogenic
ERBB2	Chr17: 39723335	NM_001382804	A	C	p.I379L	Nonsynonymous (SNV)		Uncertain significance
PAX6	Chr11: 31789913	NM_001368929	TT		p.K244Gfs	Frameshift deletion	Heterozygous	Likely pathogenic
SFRP1	Chr8: 41265168	NM_003012	C	A	p.X315L	Stop loss	Heterozygous	Likely pathogenic
BRS3	ChrX: 1.36E+08	NM_001727	G	T	p.E264X	Stop gain	Heterozygous	Likely pathogenic

ACMG, American College of Medical Genetics and Genomics; SNV, single nucleotide variant.

A Missense Mutation of the *SRGAP1* Gene as Pathogenic Variant in the First Family

The variant filtering and prioritization steps led us to believe that a missense variant on the *SRGAP1* gene (c.1849C>T, p. R617C) likely causes the disease in the first family. The logical evidence for this finding is the following:

1. I: A risk factor for this disease has previously been identified as the candidate variation in functional investigations by He et al.[12] II: The same gene has been shown to have 2 pathogenic variants (c.447A>C, p. Q149H, and c.823G>A, p. A275T). III: The potential variant is found in the Rho domain of GAP, and the wild type of *SRGAP1* has a high degree of conservation of the amino acids

in this position. IV: Liver, colon, and prostate cancer have been linked to point mutations and deletions in this type of domain, which is also found in the *DCL1* gene, demonstrating the significance of the Rho GAPas domain in carcinogenesis.[23] Altogether,

TABLE 3. Rules for combining criteria to classify sequence variants

Classification	Criteria
Pathogenic	1 1 Very strong (PVS1) AND a. ≥1 Strong (PS1-PS4) OR b. ≥2 Moderate (PM1-PM6) OR c. 1 Moderate (PM1-PM6) and 1 Supporting (PP1-PP5) OR d. ≥2 Supporting (PP1-PP5) 2 ≥2 Strong (PS1-PS4) OR 3 1 Strong (PS1-PS4) AND a. ≥3 Moderate (PM1-PM6) OR b. 2 Moderate (PM1-PM6) AND ≥2 Supporting (PP1-PP5) OR c. 1 Moderate (PM1-PM6) AND ≥4 Supporting (PP1-PP5)
Likely pathogenic	1 1 Very strong (PVS1) AND 1 Moderate (PM1-PM6) OR 2 1 Strong (PS1-PS4) AND 1–2 Moderate (PM1-PM6) OR 3 1 Strong (PS1-PS4) AND ≥2 Supporting (PP1-PP5) OR 4 ≥3 Moderate (PM1-PM6) OR 5 2 Moderate (PM1-PM6) AND ≥2 Supporting (PP1-PP5) OR 6 1 Moderate (PM1-PM6) AND ≥4 Supporting (PP1-PP5)
Benign	1 1 Stand-alone (BA1) OR 2 ≥2 Strong (BS1-BS4)
Likely benign	1 1 Strong (BS1-BS4) and 1 Supporting (BP1-BP7) OR 2 ≥2 Supporting (BP1-BP7)
Uncertain significance	Variants should be classified as uncertain significance if other criteria are unmet or the criteria for benign and pathogenic are contradictory.

by the ACMG guideline, the variant is included in the PS3 category of the criteria for the classification of variants (**TABLE 4**).

2. The following population databases show the frequency of the candidate variant in the heterozygous state: Iranome: 0.006875, 1000 Genome: 0.00060, GnomAD: 0.0005, EXAC: 0.00110. Due to how rare the desired variant is in population databases, the ACMG guideline puts it in the PM2 category of variant categorization criteria (**TABLE 4**).
3. Some bioinformatics tools categorize this variant as harmful (**TABLE 5**). As a result, it is listed in the PP3 category in the classification of variants according to ACMG.
4. Two members of this family who have FNMTC have the *SRGAP1*: NM_020762:c.C1849T variant, according to the analysis of Sanger sequencing data. This indicates that the targeted variant was present in these 2 individuals (III-1 and III-6). Moreover, they are heterozygous at the mutational site. On the other hand, this region of the sequence has not changed in any of the 2 healthy individuals (III-2 and IV-4) nor have any of their potential variants. However, the person IV-5 is a carrier for the required substitution and heterozygous for the proposed variant. **FIGURE 4B** shows the outcomes of Sanger sequencing for this family. Based on the cosegregation results in this family, the ACMG guideline places this variant in the PP1 category (**TABLE 4**) for variants.

Altogether, this variant (c.1849C>T, p. R617C) meets the requirements for PS3, PM2, PP1, and PP3 classification under the ACMG guideline and is classified as probable pathogenic under the rules governing the combination of evidence for that classification (**TABLE 3**).

TABLE 4. Criteria for classifying pathogenic variants

Evidence of pathogenicity	Grouping
Very strong evidence of pathogenicity	PVS1: Null variant (nonsense, frameshift, canonical +/–1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function (LOF) is a known mechanism of disease
Strong evidence of pathogenicity	PS1: Same amino acid change as a previously established pathogenic variant regardless of nucleotide change PS2: De novo (both maternity and paternity confirmed) in a patient with the disease and no family history PS3: Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product PS4: The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls
Moderate evidence of pathogenicity	PM1: Located in a mutational hot spot and/or critical and well-established functional domain (eg, active site of an enzyme) without benign variation PM2: Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes, or EXAC PM3: For recessive disorders, detected in trans with a pathogenic variant PM4: Protein length changes due to in-frame deletions/insertions in a nonrepeat region or stop-loss variants PM5: Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before PM6: Assumed de novo, but without confirmation of paternity and maternity
Supporting evidence of pathogenicity	PP1: Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease PP2: Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease PP3: Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc) PP4: Patient's phenotype or family history is highly specific for a disease with a single genetic etiology PP5: Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation
Stand-alone evidence of benign impact	BA1: Allele frequency is above 5% in Exome Sequencing Project, 1000 Genomes, or EXAC
Supporting evidence of benign impact	BP1: Missense variant in a gene for which primarily truncating variants are known to cause disease BP2: Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in cis with a pathogenic variant in any inheritance pattern BP3: In-frame deletions/insertions in a repetitive region without a known function BP4: Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc) BP5: Variant found in a case with an alternate molecular basis for disease BP6: Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation BP7: A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved

EXAC, Exome Aggregation Consortium.

FIGURE 4. **SRGAP1 protein structure and Sanger sequencing results in family 1. A, Schematic map showing the location of the mutation in the protein SRGAP1. This mutation occurred in exon 16 of this gene and caused the amino acid arginine to change to cysteine (R617C). B, Sanger sequencing confirmed the substitution c.1849C>T, marked in green. This substitution is found within exon 16 of the *SRGAP1* gene in both people with papillary thyroid cancer (III-1, III-6) as well as the absence of it is evident in 2 healthy members of the family (III-2, IV-4). Individual (IV-5) also inherited the candidate variant as a heterozygote.**

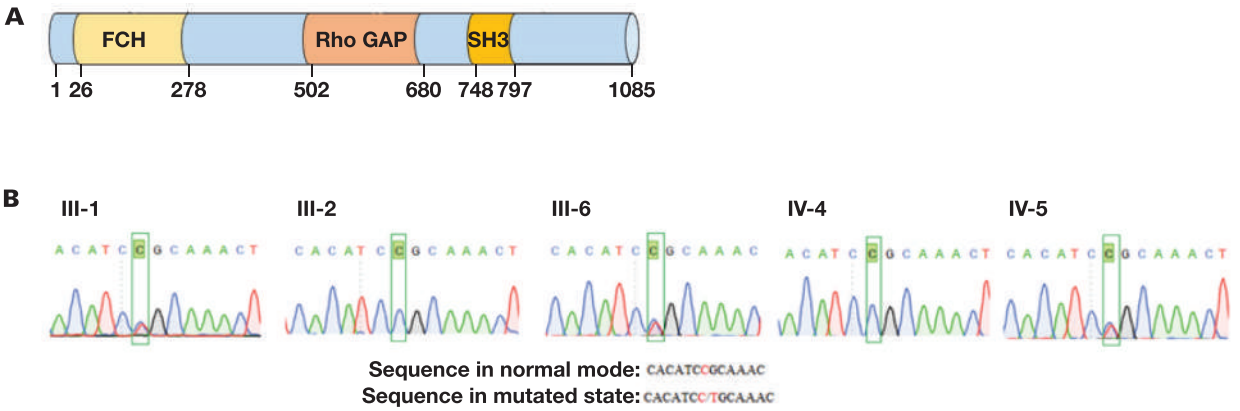

TABLE 5. **Classification of the variant p. R617C as destructive by bioinformatics tools**

Pathogenicity prediction tools	SIFT	PolyPhen-2	Mutation Taster	FATHMM	PROVEN	M-CAP
p.Arg617Cys	Damaging	Probably damaging	Damaging	Damaging	Damaging	Damaging

The candidate variant for the first family is a missense variant leading to a cysteine-to-arginine substitution at position 617. This mutation is directly located in the Rho GAP domain, involved in nearly all of the essential cellular processes.[24] The *SRGAP1* coding gene produces a protein with 1085 amino acids and several functional domains (an N-terminal F-BAR domain, a Rho GAP domain, and a C-terminal SH3 domain) as its biggest transcript. The intermediate interacts with other proteins and participates in several activities, including cell migration inhibition, decrease, negative regulation, controlling the activity of enzymes, modifying the intracellular localization of signaling pathway elements, and mediating the formation of multiprotein complexes (**FIGURE 4A**). This GAP protein is specific to cell division cycle 42 (Cdc42), which is expressed during nervous system development. SRGAP proteins generally interact with Robo1, the transmembrane receptor of slit proteins, through Rho GAPs. Slit proteins are secreted proteins that regulate leukocyte and neuronal migration as well as axon guidance.[25]

In 2013, He et al[12] identified the tumor suppressor gene *SRGAP1* as a risk factor for FNMTC. Using a genome-wide association study (GWAS), they sequenced all of the *SRGAP1* exons and the edges between exons and introns in the people in the affected families with FNMTC. Last, in the same FNMTC family, 4 germline missense variants (H875R, Q149H, A275T, and R617C) were linked to a PTC phenotype. Functional tests have demonstrated that *SRGAP1* encodes a protein that inactivates Cdc42, a primary function that the Q149H and R617C variants severely compromise. These 2 *SRGAP1* mutations can lead to loss of function, which may affect Cdc42 activity. Many signaling pathways are mediated by Cdc42, which also serves as a convergence site for signal transduction in intracellular signaling networks.[12]

Variant Analysis in the Second Family

WES data analysis and variant prioritization on the data from the second family showed that the variant *FOXE1*: NM 004473: c.531-532insCGCGA

TABLE 6. **Classification of the variant p. A178Rfs*191 as destructive by bioinformatics tools**

Pathogenicity prediction tools	Metal R	FATHMM	PimateAL	Uniprot variants
p.A178Rfs*191	Damaging	Damaging	Damaging	Pathogenic

was likely to be a pathogenic variant when compared with the gene panels for thyroid cancer and several other malignancies that had affected members of the second family.

Interpretation of (c.531_532insCGCGA: p. A178Rfs*191) according to ACMG criteria is as follows:

1. The candidate variant was thought to be a likely pathogen by Varsome's database, and this frameshift variant of the *FOXE1* gene causes the loss of function, which is a reason for the disease. Moreover, the pathogenic mutation c.743C>G, p. A248G, in the same gene has been identified as the disease-causing variant. The ACMG recommendations for categorizing variants according to their pathogenicity (**TABLE 4**) classify this variant as PVS1.
2. In terms of population database frequency, this variant is not found in GnomAD and according to ACMG guidelines, is classified as PM2 (**TABLE 4**).
3. The *FOXE1* gene is involved in thyroid morphogenesis and has a forked domain that functions as a thyroid transcription factor. This variant was identified as potentially harmful and pathogenic using some bioinformatics tools and classified in the PP3 category (**TABLES 4** and **6**).

All of the evidence (PVS1, PM2, and PP3) suggests that the c.531_532insCGCGA: p. A178Rfs*191 variant is likely to be pathogenic

FIGURE 5. **The result of the electropherogram obtained from Sanger sequencing in the proband (III-1) of family 2. Sanger sequencing had not confirmed this variant.**

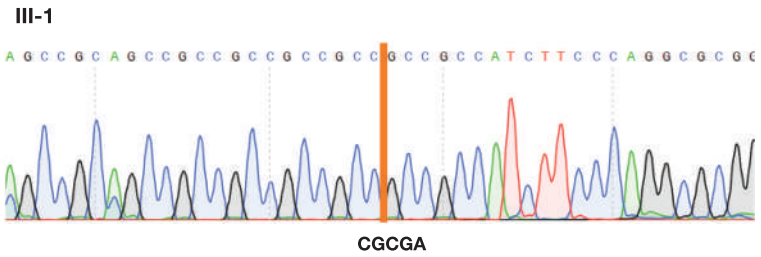

TABLE 7. **Summary of numerous studies that used various approaches to look at the genetic component of FNMTC in families with nonsyndromic FNMTC**

Chromosomal position	Loci/gene	Variant	Odds ratio	Population	Reference
		Linkage analysis			
1q21	fPTC/PRN1		3.58	American	28
2q21	NMTC1		4.17	Tasmanian	29
6q22		rs12183572	3.3	American and Italian	30
8p23.1-p22	FTEN		4.41	Portuguese	31
8q24	AK023948		1.3		32
12q14	SRGAP1	p.Q149H,p.A275T,p.R617C	1.21	Ohio and Poland	12
14q32	MNG1		3.8	Canadian	33,34
		Linkage analysis and NGS			
4q32	Enhancer associated with POU2F1 andYY1	4q32A>C		Caucasian	35
11q22.3	ATM	p.P105Rp.,A1309T			36
16p13.3	SRRM2	p.S346F			37
19p13.2	TCO/MYOF1	p.G134S	2.97	French	38,39
20p12.3	PLCB1		3.01	Caucasian	40
		GWAS			
2q35	DIRC3	rs966423,rs11693806	1.34,1.433	Iceland, European	41
8p12	NRG1	rs2439302,rs2466076	1.36,1.32	Iceland	41,42
9q22.33	FOXE1	p.A248G	1.75	Portuguese	43,44
9q22.33	PTCS2	rs965513	1.6-1.9	European, Japanese, Belarusian	43,44
9q22.33	MYH9				45
14q13.3	NKX2-1	p.A339V	1.37	Iceland, Columbus, Spain	43
		NGS			
1q41	BROX1				46
4q21.21	ANXA3	p.Y157S		Brazilian	47
7q31.33	POT1	p.V29L			48
15q23	MAP2K5	p.A321T,p.M367T			49
19q13.33	NOP53	p.D31H			50
22q12.1	CHEK2	p.E239K		Italian	51
		Other methodology			
	miR-886-3p and *miR-20a*				52
14q12	TINF2	p.W198fs			53
19p13.2	TIMM44				54
19p13.11	GRIM-19				55

FNMTC, familial nonmedullary thyroid cancer; GWAS, genome-wide association study; NGS, next-generation sequencing.

(**TABLE 4**). However, Sanger sequencing did not confirm the variant. **FIGURE 5** shows the electropherogram output of the Sanger.

The limitations of this study can be mentioned as follows: (1) finding a suitable sample (diagnosed nonsyndromic nonmedullary cancer; the disease has occurred in at least 2 generations of the same family; at least 2 of the affected members in the family pedigree are less than 50 years old when the disease occurs; medical documents of the family members are enough); (2) the high cost of the WES test and the time-consuming interpretation of the results, considering that there is no specific gene panel for FNMTC; (3) inconsistency in the results of (WES) with the results of Sanger sequencing (of the limitations of WES[26]). This issue can be due to the occurrence of mutations in GC-rich regions, mutations related to CNVs, or epimutations. In families like family 2, due to the early onset of cancer in the family, the multiple affected members, and the multigenerational transmission of this disease, there is a high probability of a genetic condition predisposing to cancer in this family. In addition to the cases mentioned, other potential causes for determining a genetic factor in this family include intronic variants and unknown new genes. Identifying new variants in WES tests of people with FNMTC helps us screen other patients, and it also allows us to create a diagnostic panel for patients with this cancer according to the demographics of our country. On the other hand, considering that somatic mutations can be a possible cause of thyroid cancer in the first family, we suggest that in future studies on the proband's tumor tissue in the first family a WES test be performed.

Along with the increase in NMTC cases in recent years, the familial subtype of the disease has also become more common than previously believed. However, the genes responsible for the great majority of nonsyndromic FNMTC cases are still unknown. Although the heritability of thyroid cancer is strongly supported, only a limited subset of the genetic variants has been definitively linked to an elevated risk for this malignancy. Rare highly penetrant or frequent low-penetrant variants may occasionally cause thyroid cancer's high heritability.[27]

The most significant genes related to increasing the risk of FNMTC have been presented in **TABLE 7**. Incorporating these findings into patient clinical data would lead to early detection of the disease and more efficient treatment of the cases. Discovery of additional familial NMTC risk genes could help to develop population-based prevention strategies and individualized patient care, a valuable achievement that is essential, given the severe risk of getting cancer.

Linkage analysis was used in the early studies of FMNTC to find several different loci,[28,29,31,33,38,56] but no single gene was linked to the condition. Also, Sanger sequencing, which shows genetic diversity and multigenic and multifactorial inheritance, has been used many times to look into FNMTC, but no conclusive data were found.[36] Furthermore, GWAS suggested that different groups have various thyroid cancer-related SNPs. For instance, 2 GWAS loci were only replicated in the Italian population and not in the Polish or Spanish populations, according to the research of Figlioli et al.[57] This demonstrates that the GWAS for thyroid cancer can vary depending on the study. Many of these findings support the hypothesis that various populations may have varying genetic compositions.

Conclusion

The advancement of NGS technologies has not only led to the discovery of novel candidate variants and a significant improvement in our understanding of FNMTC susceptibility but it has also made it simpler to locate new predisposing genes. As a result, gene testing has become quicker and less expensive. However, due to the complexity of the entire analytical and validation process, methodologies and results must be taken with caution.

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Data Availability

On reasonable request, the corresponding author will provide data that support the study's findings.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Anti-E alloimmunization from a platelet apheresis transfusion in a 22-month-old male with acute myeloid leukemia

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Key words: anti-E; apheresis platelets; blood banking/transfusion medicine; RBC; alloimmunization; pediatric transfusion

Abbreviations: RBC, red blood cell; DAT, direct antiglobulin test

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ABSTRACT

RhD alloimmunization from platelet transfusions have been documented in the literature. However, non-RhD platelet alloimmunization is much less frequent and the risk for non-RhD alloimmunization from platelets is thought to be extremely low and most associated with buffy coat pooled platelets. A 22-month-old male with acute myeloid leukemia received 99 mL apheresis platelets for thrombocytopenia. Three months later, an antibody screen, the direct antiglobulin test (DAT), and red blood cell (RBC) genotype were sent for laboratory evaluation. The antibody screen was positive, with anti-E identified. The DAT was negative and the RBC genotype of the patient was predicted to be negative for the E antigen whereas the platelet donor was predicted to be positive for E antigen. There is a risk of alloimmunization of non-RhD antigen from platelet pheresis transfusion even in a patient less than 2 years old.

Clinical History

A 22-month-old male with a medical history of trisomy 21 presented to his primary care physician with a petechial rash, fever (100.6°F), mild cough, and congestion. He subsequently was found to have thrombocytopenia (platelets = $36 \times 10^3/\mu L$) and leukopenia (white blood cells = $10.7 \times 10^3/\mu L$, peripheral blast = 2%). The patient was admitted to our institution with concern for a diagnosis of acute leukemia. Prior to a bone marrow biopsy, 10 mL/kg of group O Rh-positive apheresis platelets were ordered, with 99 mL total given due to the petechial rash and a risk of bleeding. The platelet unit was collected as a large volume delayed sampling (LVDS) in platelet additive solution. During the transfusion, the patient's temperature increased from 98.1°F to 100.1°F and acetaminophen (15 mg/kg) was given orally, with temperature drop to 99°F. No transfusion reaction was called at the time, which may have been due to neutropenic fever. In our institution, calling a transfusion reaction is at the discretion of the treating physician. The bone marrow biopsy showed mildly increased blasts in bone marrow smears but no evidence of acute leukemia. To consider the diagnosis of an autoantibody, a direct antiglobulin test (DAT) and type and screen were performed. The patient typed blood group O Rh positive with a negative DAT. The patient was discharged and closely monitored. A respiratory viral panel was performed as an inpatient test and was initially negative but repeated 6 days later and was positive for adenovirus and parainfluenza 3. The top differential etiologies of the cytopenia were viral associated thrombocytopenia and early leukemia.

Three months later, the patient presented to his primary care physician with moderate to severe fatigue and thrombocytopenia ($40 \times 10^3/\mu L$). Flow cytometry was performed on the peripheral blood and showed increased blasts present. Subsequently a second bone marrow biopsy was performed, revealing acute myeloid leukemia associated with Down syndrome. The patient was admitted for induction chemotherapy with cytarabine, daunorubicin, and dexamethasone, and prior to Hickman catheter placement, 1 unit of platelets was ordered, and type and screen were performed.

Clinical and Laboratory Information

The patient's blood group was confirmed to be group O Rh positive. Commercial red cell antibody screen (Ortho Clinical Diagnostics) and identification panels were positive. Plasma gel testing (37°C) was performed and identified the presence of an anti-E antibody. A DAT was performed and was negative. Genotype testing was performed using a polymerase chain reaction–based kit to evaluate the presence of common Rh blood group alleles. It showed the patient was predicted to be C+,c-,E-,e+. This was consistent with a new alloimmunization-anti-E immunoglobulin G antibody identified. He received 99 mL of E antigen negative platelets without complication. He received induction chemotherapy during admission and

E antigen negative red blood cell (RBC) and platelet transfusions without complications and was discharged on hospital day 10.

The newly identified anti-E antibody proposed the possibility of alloimmunization following the apheresis platelet transfusion 3 months prior or, rarely, a naturally occurring anti-E.[1-4] To further investigate a serologic transfusion reaction, an investigation into the donor of the previously transfused platelets was requested from the American Red Cross.

The donor of the initial transfusion of the apheresis platelet unit was contacted by the American Red Cross to obtain a follow-up sample. The subsequent serologic work-up and RBC genotype evaluation determined that the donor was blood group O Rh positive and E antigen positive. This makes the alloimmunization to the E antigen following platelet transfusion more plausible.

Discussion

We did a PubMed search of RBC alloimmunization in pediatric patients after platelet transfusions and found 1 article published in the *Transfusion Journal*.[5] In another article, Haspel et al[6] described a case of a young infant who received less than 0.6 mL of D+ RBCs from platelet transfusion and developed anti-D. This is the first occurrence of a 22-month-old pediatric patient with hematologic malignancy and no RBC transfusion history developing a clinically significant anti-E antibody after receiving a pediatric dosage of leukoreduced apheresis platelets from an E antigen–positive donor.

Platelets express antigens A and B in low dosage but do not express the Rh antigens (D, C, c, E and e) on the platelet membrane.[7] It is thought the risk for alloimmunization is linked with the residual RBCs in platelets.

RhD alloimmunization from platelet transfusions is documented in the literature to have 0% to 7.8% incidence.[8] Non-RhD platelet alloimmunization is much less frequent and the risk for non-RhD alloimmunization from platelets is thought to be 0.7%[9] and most commonly associated with buffy coat pooled platelets, not platelet apheresis. Additionally, in most of the cases found in the literature, the recipients also had a history of previous RBC transfusion prior to alloimmunization.

There is evidence of alloimmunization of anti-E antibody following platelet transfusions.[10] A 5-year study from 2007 to 2011 based on a French hemovigilance database by Moncharmont et al[11] followed transfusion reactions after platelet transfusion (apheresis platelet concentrate and pooled platelet concentrate). Anti-E was the most common alloantibody identified, followed by anti-D. The other common non-RhD antibody specificities were anti-C, anti-c, and anti-e.

The increased alloimmunization from buffy coat pooled platelets to apheresis pooled platelets seems to be related to the amount of residual RBCs, 0.03 mL to 0.6 mL in buffy coat platelets compared with 0.00017 mL to 0.009 mL in apheresis platelets.[11] In this case, our patient received a 10 mL/kg dose (99 mL). This is half the normal platelet unit of the adult dose of 200 mL, making the suspected residual RBC much less in the apheresis platelet transfusion our patient received.

It has been shown that neonates and young children have significantly decreased alloimmunization rates with RBC transfusions than adults.[12] Current literature cites the alloimmunization frequency in the pediatric population at 1.5%.[13] Literature on Rh D RBC alloimmunization in the pediatric population is absent when using apheresis and leukoreduced platelets,[5] even when giving Rh positive platelets to Rh negative recipients.

Alloantibodies such as anti-E and anti -M have been shown to be identified in patients who have no prior stimuli, such as previous pregnancy or transfusions.[1] Some examples of these "natural occurring" antibodies can be detected in "enzyme only" treated cells[14,15] and are thought to be induced from exposure to environmental agents such as bacteria.[3] In our case, the platelet donor being E antigen positive supports the possibility that E antigen exposure was associated with the alloimmunization.

Immunosuppression such as that secondary to malignancies themselves or chemotherapy has not been shown to reduce the rate of alloimmunization.[16] It has been shown that in cases of certain infections and increased inflammatory states, there can be an increased risk for alloimmunization[17]; as seen in the current case, the patient had an initial presentation of an upper respiratory infection.

A recent study demonstrated lack of alloimmunization in neonates and children up to 3 years of age[12]; however, children can develop blood group alloantibodies, as seen in the current case. Tamai et al,[18] in their retrospective, multicenter, nationwide cohort study using data recorded in 50 participating medical facilities in Japan, found that alloimmunization did not occur from RBC transfusions within the first month of life and rarely occurred (0.46%-0.80%) after transfusion within the first decade of life. In a letter to the editor by Moncharmont et al,[8] the authors argue that better antibody screening for RBC alloimmunization after platelet transfusion with detection of new RBC antibodies partly explains the improvement in notification to hemovigilance systems, and monitoring RBC alloimmunization after platelet transfusion must be maintained during pooled platelet concentrate transfusion due to the risk of alloimmunization.

Conclusion

To our knowledge, this is the first report of alloimmunization to the E antigen from apheresis platelet transfusion of a child under the age of 2 years with no prior transfusion history from RBCs or apheresis platelets. It will be prudent to suspect alloimmunization from platelet transfusions in a child with no history of RBC transfusions. If a new antibody is detected, the platelet donor should be phenotyped for the cognate antigen.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Heidenhain variant of Creutzfeldt-Jakob disease masquerading as neuromyelitis optica spectrum disorder: recognizing when apheresis is not the answer

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Key words: Heidenhain variant, Creutzfeldt-Jakob disease, neuromyelitis, optica, transfusion medicine

Abbreviations: NMO, neuromyelitis optica; AQP4, aquaporin 4; NMOSD, neuromyelitis optica spectrum disorders; CJD, Creutzfeld-Jacob disease; PrP^C, normal host-encoded cellular prion protein; PrPSc, misfolded form of host-encoded cellular prion protein; NMDA, N-methyl-D-aspartate; VGCC, voltage-gated calcium channel; GAD65, anti–glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; DPPX, dipeptidyl-peptidase–like protein 6; CRMP5, anti-CV2/collapsin response mediator protein 5; CASPR-2, anti–contactin-associated protein-like 2; TPE, therapeutic plasma exchange; ACE, angiotensin-converting enzyme inhibitor; ACD-A, anticoagulant citrate dextrose solution, solution A; RT-QuIC, real-time quaking-induced conversion; RR, reference range

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ABSTRACT

The Heidenhain variant of Creutzfeld-Jakob disease (CJD) is a rare form that initially presents with visual disturbances. In early stages, the presentation can mimic neuromyelitis optica spectrum disorders (NMOSD) and lead to unnecessary treatment modalities. Herein, we describe a case of a 66-year-old man who presented with bilateral vision loss and retro-orbital discomfort. In addition to immunosuppressive therapy, he received 4 rounds of therapeutic plasma exchange after his preliminary diagnosis of NMOSD. We were surprised to note that his condition did not show improvement but deteriorated, with severe neurocognitive symptoms. Eventually, CJD was suspected, and real-time quaking-induced conversion (RT-QuIC) was performed. By the time the diagnosis of Heidenhain variant of CJD was confirmed, the patient was discharged to hospice care and died shortly after.

Introduction

Neuromyelitis optica (NMO) is an inflammatory autoimmune disorder that was historically considered a variant of MS.[1,2] However, autoantibodies against aquaporin 4 (AQP4) were discovered to be a highly specific marker for NMO. This specificity differentiates NMO from MS and has broadened the clinical and imaging spectrum of NMO. In turn, the classification of NMO spectrum disorders (NMOSD) was coined to incorporate individuals with AQP4-IgG antibodies but with limited forms of NMO.[1] NMOSD also encompasses the cerebral, diencephalic, and brain-stem lesions that occur in a small subset of patients who otherwise have typical NMO features.[1]NMOSD often presents with severe bilateral and recurrent optic neuritis or severe longitudinally extensive transverse myelitis.[3] Various conditions including MS, acute disseminated encephalomyelitis, sarcoidosis, Sjögren syndrome, systemic lupus erythematosus, primary CNS lymphoma, Behçet disease, Leber hereditary optic neuropathy, syphilis, herpes viruses, tuberculosis, and Lyme disease have been reported to mimic NMOSD.[3] Distinguishing among these conditions is critical for appropriate diagnosis and therapy.

One disorder that has not been reported to mimic NMOSD is Creutzfeldt-Jakob disease (CJD).[4] This neurodegenerative condition is uniformly fatal and most commonly occurs sporadically. Like other prion diseases, its pathological characteristics arise from conversion of normal host-encoded cellular prion protein (PrP^C) to a misfolded form (PrP^{Sc}) by posttranslational modification.[5]

Although there are multiple variants of CJD that may manifest differently, one of the rarest is the Heidenhain variant, which occurs in ~5% of CJD cases.[4] This condition is characterized by visual disturbances, which can be particularly distressing for patients because these visual disturbances often take the forms of hemianopia or scotoma, misperceptions, hallucinations, distortions, and palinopsia.[4] The progression of these symptoms is rapid and may lead to the development of cortical blindness.[6,7] Due to the predominance of visual symptoms, patients may be seen by optometrists or ophthalmologists for their initial evaluation. Thus, awareness of this uncommon condition is critical. Herein, we present the case of a patient initially diagnosed with NMOSD, who had initially received immunosuppressive therapy and plasma exchange without health improvement but was eventually diagnosed with CJD.

Case Presentation

A 66-year-old man with a medical history significant for cataracts, nonischemic cardiomyopathy, diabetes mellitus type 2, and hypertension was admitted after 1 month of progressive bilateral vision loss and retro-orbital discomfort. Before these symptoms, the wife of the patient reported that he had experienced approximately 8 to 10 months of irritability, increased confusion, and inattention.

The results of neurological examination were notable for bilateral nonreactive pupils, mild right abduction deficit, and normal optical discs and retina. Computed tomography angiography revealed irregular optic nerve sheaths. Magnetic resonance imaging (MRI) showed mild cerebral volume loss and microvascular disease but no restricted diffusion, hemorrhage, or midline shift. There was slight asymmetric and irregular left perioptic nerve sheath enhancement and questionable left optic nerve enhancement. There was no abnormal enhancement within the brain parenchyma or meninges. A few scattered small FLAIR hyperintensities in the periventricular and subcortical white matter were thought to be nonspecific and suggested sequelae of microvascular disease, with MS not mentioned in the differential diagnosis. Spinal imaging did not reveal any white matter or gray matter changes. Abdominal and pelvis imaging results were unremarkable. Visual acuity was reported as 20/200 on the right and 20/150 on the left.

Laboratory evaluation performed to exclude other causes, such as MS, CNS lymphoma, and immune and infectious causes of encephalopathy, yielded unrevealing results. Negative findings were recorded for cerebrospinal fluid (CSF) cytology and flow cytometry testing, oligoclonal bands, CSF and blood cultures for bacteria and fungi, and infectious disease testing for herpes simplex virus, varicella-zoster virus, West Nile virus, human T-cell lymphotropic virus type 1 and 2, HIV, *Treponema pallidum, Borrelia burgdorferi, Mycobacterium tuberculosis*, and *Bartonella* species.

Immunologic and rheumatologic testing results were unrevealing, including normal serum and urine protein electrophoresis and immunofixation results, and negative rheumatoid factor and antinuclear antibodies results. Antibodies uniformly tested negative for the following targets: SS-A/SS-B, aquaporin 4, myelin oligodendrocyte glycoprotein, acetylcholine receptor, N-methyl-D-aspartate (NMDA) receptor, anti-neutrophil cytoplasmic, voltage-gated calcium channel (*VGCC*), Purkinje cell cytoplasmic types 1, 2 and Tr, anti-$GABA_A$ and $GABA_B$ (gamma aminobutyric acid) receptor, anti–glutamic acid decarboxylase (GAD65), glial fibrillary acidic protein (GFAP), dipeptidyl-peptidase–like protein-6 (DPPX), anti-CV2/collapsin response mediator protein-5 (CRMP5), anti-contactin-associated protein-like 2 (CASPR-2), anti-glial nuclear, amphiphysin, LGI1, and neurochondrin. These negative results put most of the paraneoplastic syndromes, autoimmune diseases including autoimmune optic neuropathy, neurodegenerative diseases, and autoimmune vasculitis lower on the list of differential diagnoses.

Given the symptoms, the age of onset, and imaging findings, the main concern was for NMOSD. MS was, again, lower on the differential diagnosis list because the age of onset would have been earlier. Also, the imaging of more than 9 T2 lesions, a diagnostic clue for optic neuritis, was not present in our patient. The patient was treated with 5 days of IV methylprednisolone without significant change in his condition, followed by therapeutic plasma exchange (TPE). Per the American Society for Apheresis guidelines, optic neuritis is a category II indication for TPE, meaning that apheresis is accepted as "second-line therapy, either as stand-alone treatment or in conjunction with other modes of treatment."[2]

The apheresis team recommended 5 one plasma volume exchange procedures during the next 10 days. As the patient was taking an angiotensin-converting enzyme (ACE) inhibitor, which poses a risk of bradykinin-mediated severe hypotensive reactions in the setting of TPE, the apheresis service recommended medication discontinuation and a 48-hour washout period before TPE initiation. In the interim, the patient was discharged from the hospital and began TPE as an outpatient. He ultimately underwent 4 TPE procedures on an every-other-day schedule (excluding weekends), utilizing 5% albumin as the replacement fluid and anticoagulant citrate dextrose solution, solution A (ACD-A) as the anticoagulant. However, there was no improvement in his condition, and his health continued to decline, with altered mental status, progressive visual disturbance, and worsening movement ability during a 1-week period. Before his fifth TPE procedure, the patient was readmitted to the hospital, and TPE was postponed.

By that time the patient demonstrated confabulation, inappropriate emotions, bilateral extremity paratonia, and hyperreflexia. His left pupil was sluggish and nonreactive, whereas his right pupil was briskly reactive. A positive Babinski sign and myoclonus was noted. An electroencephalogram (EEG) revealed right posterior quadrant slowing, left hemispheric voltage attenuation, mild to moderate generalized slowing, and multiple events of bilateral foot movements mostly in drowsiness or sleep, at times rhythmic and without ictal correlation. Repeat MRI demonstrated hemispheric cortical ribboning greater on the right than the left, thalamic hyperintensity (pulvinar sign), and no enhancement.

The differential diagnosis was concerning for the Heidenhain variant of CJD, particularly with the severe visual disturbance. On further review, there was no history of travel, blood transfusion, transplantation, injections/substance use, or family history of CJD or other neurodegenerative disorders. CSF examination results revealed normal protein, glucose, and electrolyte levels. We sent a CSF specimen from the patient to the National Prion Disease Pathology Surveillance Center in Cleveland, OH; the findings from that center included a real-time quaking induced conversion (RT-QuIC) result for T-tau protein of 1901 pg/mL (reference range [RR], 0-1149 pg/mL), and 14-3-3 gamma of 17,735 AU/mL (RR, <30-1999 AU/mL), both of which are consistent with a diagnosis of CJD. While awaiting these results, the patient was transferred to home hospice care and died 1 month after his initial hospital admission.

Discussion

This rare case of CJD was initially misdiagnosed as NMOSD. Although to our knowledge the eventual outcome could not have been altered for our patient, the significance of earlier recognition of the disease and its outcome should not be overlooked. CJD is uniformly fatal, with no effective treatment; still, understanding the etiology of patient symptoms and health deterioration is important for improving patient, family, and caregiver comfort. Because prion diseases are transmissible, early diagnosis is crucial to protect against transmission during medical care. Although 90% of CJD cases are sporadic, the diagnosis has implications for family members because hereditary forms occur; definitive elucidation of the particular CJD type is only possible through tissue and genetic testing.

Often, the predominant symptoms depend on the particular type of CJD. The classical early signs and symptoms may include visual or auditory disturbances, seizures, isolated psychiatric manifestations, atypical parkinsonian syndromes, pseudobulbar syndrome, isolated

involuntary movements, acute or subacute onsets mimicking a stroke, isolated aphasia, and neuropathic manifestations.[6] The Heidenhain variant of CJD is characterized by a short disease duration, marked visual impairment as the primary symptom, and subsequent neurologic manifestations.[8] Patients experience visual field restriction, blurred vision, vision loss, and even cortical blindness.[9] Other visual symptoms may include disturbed perception of colors or structures, optical hallucinations, and optical anosognosia (Anton syndrome).[9,10]

Although the initial focus is on visual symptoms, additional neurologic signs and dementia develop as the disease progresses, which are important clues if NMOSD is in the differential diagnosis. NMOSD tends to include high GFAP levels; however, this was not so in our patient. Neuropathological examinations revealed severe damage in the occipital cortex. We note that limbic-system involvement and basal-ganglia damage in our patient were less pronounced, compared to findings in patients with other CJD subtypes. Differentiating other rapidly progressing types of dementia requires an extensive workup to eliminate infectious, vascular, autoimmune, malignant, and toxic-metabolic causes.[11]

Diagnosing the Heidenhain variant of CJD can be challenging, particularly when visual symptoms are the only initial clinical manifestation. The differential diagnosis includes other neurocognitive diseases, such as depression, vascular dementia, normal-pressure hydrocephalus, and encephalitis.[12] Although optic symptoms could be seen in patients with MS, having more than 9 T2 lesions seen on MRI is specific to MS,[13] and this finding was absent in our patient. Several diagnostic modalities aid in the identification of this CJD variant. EEG often shows periodic sharp wave complexes. CSF analysis reveals elevated levels of neuron-specific enolase and the presence of the 14-3-3 protein, which are surrogate markers for CJD.[10] MRI is crucial in confirming the diagnosis by demonstrating cortical ribboning in the visual cortex[8] and increased signal intensity in basal ganglia.[10,14]

The development of a more specific test in 2010 called the RT-QuIC has improved antemortem laboratory testing for CJD.[15] RT-QuIC measures the ability of PrPSc found in the CSF to induce conversion of normal PrP to the misfolded form in real time, by monitoring the amount of fluorescent dye to which it binds itself. It has a reported sensitivity for sporadic CJD of ~90% and specificity of 99%-100%.[15] Histologically, CJD shows spongiform degeneration, gliosis, and nerve-cell loss, which are particularly severe in the occipital lobe in this variant.[10] Combining these diagnostic tools and multidisciplinary management increases the likelihood of identifying the Heidenhain variant and differentiating it from other disorders with similar clinical presentations.[16]

The diagnosis of CJD was delayed in our patient. The primary symptom of acute onset vision loss with questionable imaging findings led to a presumptive diagnosis of NMOSD, for which the patient was treated with corticosteroids and TPE. The imaging signs of CJD were not initially apparent, which is a not-uncommon finding in this disorder.[17] However, when repeat imaging was performed after no clinical improvement, the cortical ribbon sign and signal hyperintensity (pulvinar sign) led us to consider the Heidenhain variant of CJD, which was eventually confirmed by specific CSF testing. We note that some patients with CJD may have depression and personality change early in their disease course, with eventual visual hallucinations (as in our patient) or aggression.[4,18]

Conclusion

Recognition of the Heidenhain variant of CJD is essential for appropriate management and counseling. Although no cures or therapies currently exist to slow disease progression, symptom mitigation and comfort care should be introduced, particularly for psychiatric symptoms, as well as for tremors, spasms, and pain. Clinicians should consider CJD in patients presenting with visual symptoms, especially when accompanied by neurologic signs and/or dementia, as in our patient. This CJD variant may be confused with NMO-type disorders when the only symptom is vision change; however, further evaluation should be prompt when additional symptoms cannot be cleanly attributed to existing damage.[8] The diagnosis should also be reevaluated when plasma exchange does not show the expected improvement for NMO-spectrum diseases. EEG, CSF analysis, and MRI serve as valuable tools for timely diagnosis, to facilitate supportive care and to avoid unnecessary treatment.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Clinical management of a patient following a granulocyte transfusion from a donor positive for COVID-19

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Key words: blood donor center; COVID-19; granulocyte collection; granulocyte transfusion; postdonation information; transfusion medicine

Abbreviations: ANC, absolute neutrophil count; G-CSF, granulocyte-colony stimulating factor; PDI, postdonation information; HES, hydroxyethyl starch; NT, not tested

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ABSTRACT

Granulocyte transfusions are indicated for patients with severe neutropenia and evidence of bacterial or fungal infection who are unresponsive to standard antimicrobial therapy. With a limited expiration time of 24 hours after collection, granulocytes are often transfused before results of infectious-disease screening tests are available, and before a transfusion service can perform a risk assessment if postdonation information is provided after the collection. The case we describe herein demonstrates a clinical scenario meeting indications for granulocyte transfusion, coupled with the clinical management undertaken after the granulocyte donor disclosed a positive result for a COVID-19 self-test taken 1 day after donation. In this case, the patient did not develop new COVID-19 symptoms and tested negative for COVID-19 after transfusion of the implicated unit. These findings add to the body of evidence in the literature that COVID-19 is not transmitted via blood transfusion.

Patient History

A 23-year-old woman, who was undergoing delayed intensification treatment for Philadelphia chromosome negative B-cell acute lymphoblastic leukemia, was admitted to the hospital with severe debilitating foot pain. Admission laboratory test results were notable for pancytopenia, with a low white blood cell (WBC) count of 200 cells/μL and low absolute neutrophil count (ANC) of 100 cells/μL. To treat the severe neutropenia, the patient was immediately started on granulocyte-colony stimulating factor (G-CSF).

A few days after hospitalization, the patient experienced rapid decompensation, with disseminated mucormycosis to lung, brain, and soft tissues. Multiple antifungal medications were administered, including amphotericin B and isavuconazole. For her respiratory distress, the patient was placed on high-flow oxygen therapy. After 1 week of antimicrobial therapy, CT imaging demonstrated worsening of the lung infiltrates, and the patient continued to have neutropenia, with an ANC of 0 cells/μL and no appreciable improvement in respiratory status. The clinical team requested initiation of granulocyte transfusion, which the transfusion-medicine service reviewed and approved.

Multiple unique donors were recruited by the institution's own hospital-based blood donor center. She was transfused with the first 3 granulocyte products without incident (**TABLE 1**). The fourth granulocyte donor was a 33-year-old woman who was a repeat plateletpheresis donor. On the day of collection, the donor reported feeling healthy, and her physical examination results, including temperature, were unremarkable. Nineteen hours after collection, the granulocyte product was transfused to the patient without incident. The following day, the granulocyte donor contacted the blood establishment to report feeling unwell and having tested positive that day for COVID-19 via a self-test at home.

After receipt of this postdonation information (PDI), the blood donor center notified the transfusion medicine physician, who then notified the clinical team. Because the patient was severely immunocompromised with disseminated fungal infection, the physician treating the patient was concerned for transmission of COVID-19 by the implicated granulocyte unit. The patient was followed closely for development of new upper respiratory tract viral symptoms. Ultimately, she did not develop new symptoms that would suggest infection by COVID-19. Additionally, 4 days after the implicated granulocyte transfusion, a nasopharyngeal specimen from the donor tested negative for COVID-19 via RT-PCR testing.

After transfusion of the implicated granulocyte unit, the patient received an additional 4 granulocyte units from 4 unique granulocyte donors. After transfusion of the seventh granulocyte unit, her ANC increased from 0 to 1200 cells per μL and her respiratory status improved, with decreasing oxygen requirements. The patient received 1 final granulocyte transfusion (the eighth unit), and the granulocyte transfusions were finally discontinued when her ANC remained stably higher than 1000 cells per μL and she no longer required high-flow oxygen. The patient was discharged from the hospital in stable condition

TABLE 1. Granulocyte Transfusion With WBC and ANC

Day(s) since hospital admission	WBC (cells/μL)	ANC (cells/μL)
1	300	100
14 (granulocyte unit 1)	100	NT
15 (granulocyte unit 2)	200	NT
16 (granulocyte unit 3)	200	NT
17 (granulocyte unit 4)[a]	100	NT
21 (granulocyte unit 5)	400	0
22 (granulocyte unit 6)	700	0
23 (granulocyte unit 7)	1800	1200
29 (granulocyte unit 8)	2500	1700
50 (date of discharge)	2400	1500

ANC, absolute neutrophil count; NT, not tested; WBC, white blood cell.
[a]Granulocyte donor reported positive COVID19 self-test result and had symptoms.

on day 50 after admission, approximately 1 month after the initiation of granulocyte transfusion.

Discussion

Despite numerous advances in antimicrobial therapy, bacterial and fungal infections remain a major cause of morbidity and mortality for patients with prolonged severe neutropenia (ie, ANC <500 cells/μL). Granulocyte transfusion emerged as a treatment option for patients with neutropenia in the 1960s, with increasing utilization in the 1970s and 1980s. However, this method later became less favored due to advances in antimicrobial therapy and awareness of granulocyte transfusion reactions, including lethal pulmonary events.[1] Renewed interest in granulocyte transfusions grew with the introduction of strategies to increase the granulocyte yield from healthy donors, including administration of G-CSF and dexamethasone to donors before apheresis collection.[2]

Today, although there is no formal consensus criteria in the literature for granulocyte transfusion, the granulocyte product is generally indicated in patients with severe neutropenia (ANC <500 cells/μL) who have evidence of bacterial or fungal infection and whose condition is unresponsive to standard antimicrobial therapy.[3] Granulocytes are typically administered as frequently as every day until the ANC level recovers to >500 cells/μL or until the condition of the patient improves.[4]

Granulocytes are collected from healthy ABO-compatible donors utilizing apheresis technology. Donors must have peripheral veins that can withstand the pressure of apheresis collection. Further, selection of donors with recent negative infectious disease test results may be desirable because donor infectious disease testing may not be completed by the time the granulocyte product is transfused. This situation typically results in blood establishments contacting recent plateletpheresis donors as ideal candidates, given that they have adequate veins and recent negative infectious disease test results.

Unique considerations specific to granulocyte collection include the use of stimulating agents (G-CSF and dexamethasone) to increase granulocyte yield before the collection, as well as utilization of the erythrocyte sedimenting agent, hydroxyethyl starch (HES), to increase granulocyte yield during the collection.[2,5] Stimulating agents, along with HES, have different adverse effect profiles; therefore, taking and reviewing a careful medical history is critical in selecting appropriate donors. Furthermore, the informed consent process should discuss the risks of the procedure and any applicable medications or erythrocyte sedimenting agent required.[5,6] The interval between procedures for granulocyte collection is 2 days, for a maximum of twice in a 7-day period, per current AABB standards.[7]

Current AABB standards stipulate that the minimum yield of an apheresis granulocyte unit is 1×10^{10} cells in at least 75% of units tested.[7] Granulocytes must be stored at 20°-24°C without agitation. Although the expiration date is 24 hours from the time of collection, it is generally recommended to transfuse the product as soon as possible due to concerns for product degradation and potential loss of product efficacy.[7,8] Granulocyte products, in addition to containing a large number of granulocytes, also contain 20-50 mL of RBCs.[4] Due to this large number of RBCs in the granulocyte product, donors must be ABO-compatible, the ABO and Rh type (if Rh negative) of the granulocyte unit must be confirmed by serologic testing before transfusion, and the granulocyte unit must be serologically cross-matched with the plasma specimen from the patient before issue.[7] Irradiation is required to prevent the development of transfusion-associated graft-versus-host disease. Leukocyte reduction filters shall not be used for granulocyte products. Donor infectious disease testing may not yield results at or before the time of transfusion, due to the limited 24-hour expiration time, potentially placing the patient at risk for transfusion-transmitted infection. In this setting, transfusion services shall follow their procedure (eg, "emergency release") for issuing the product without completed infectious disease testing. The most common adverse reactions from granulocyte transfusion are fever and chills.[9] A summary of granulocyte product specifications is listed in **TABLE 2**.

The results of previous retrospective and prospective studies have suggested that the efficacy of granulocyte transfusions in the treatment of patients with neutropenia may be proportional to the granulocyte dose transfused. The RING (Resolving Infection in Neutropenia With Granulocytes) study was the first multicenter randomized controlled trial designed to address the clinical efficacy of high-dose granulocyte transfusion therapy.[10] In this study, neutropenic patients (ANC <500 cells/μL) with a proven or probable bacterial or fungal infection were randomized to standard antimicrobial therapy alone, or standard antimicrobial therapy and daily granulocyte transfusions collected from donors stimulated with G-CSF and dexamethasone. The primary endpoint was composite survival plus microbial response at 42 days. Although the results of the RING study showed that granulocyte transfusions had no effect on the primary outcome, the interpretation of the study results is limited due to low accrual rates, resulting in the study reaching low power to detect the proposed difference. In a post hoc analysis of RING data, subjects who received larger doses of granulocytes (>0.6×10^9 granulocytes/kg) appeared to have more favorable outcomes than those receiving lower doses.[11] Further, the presence of development of WBC antibodies for patients within the RING study had no demonstrable effect on the primary outcome (survival and microbial response at 42 days), suggesting no clinical concern for recipient WBC alloimmunization due to granulocyte transfusion.[12] However, there are still recommendations that patients with alloimmunization should receive HLA-matched granulocyte transfusions whenever possible.[13]

PDI encompasses any information provided by the donor or another source after a blood donation that would have resulted in donor deferral had the information been provided earlier.[14] PDI is frequently associated with the development of disease symptoms (eg, respiratory viral

TABLE 2. Granulocyte Product Specifications[7,8]

Product specification category	Product specification
Minimum yield	1×10^{10} cells in ≥75% of units tests
Storage temperature	20°-24°C
Storage conditions	Without agitation
Expiration	24 hours from time of collection
ABO compatibility requirement	Required
Irradiation requirement	Required
Leukoreduction requirement	Do not leukoreduce

symptoms) or other disclosed information (eg, medication disclosure) arising after donation. It can also arise when new donor information is disclosed at subsequent donations, which may call into question the safety of previously donated blood products.

PDI management will vary based on the type of PDI relating to the circumstance. Typically, the blood establishment will initiate the PDI management process by gathering information from the donor (or other source) and quarantining the blood product(s). If the products have been distributed, the transfusion service receiving the products is notified to determine product disposition for potential quarantine. The physician covering the transfusion service will perform an evaluation of the PDI and make recommendations regarding product disposition, notification of the physician treating the patient in question if implicated products have been transfused, and/or recall of the components from current or previous collections.

Blood establishment physicians may also make recommendations regarding donor deferral. Depending on the nature of the PDI disclosed, previously collected blood products from the donor may require evaluation ("look back"). For donors reporting developing a respiratory illness shortly after donation, blood establishments and transfusion services will typically discard the unit.

Because most routine blood products (RBC, plasma, and platelets) are not labeled for issue until infectious disease screening tests have yielded results, there is often time for the blood establishment or transfusion service to quarantine and discard the products after receipt of PDI. In the setting of granulocyte distribution, the product is most likely to have been transfused before blood establishments or transfusion services can act on PDI. The donor, in this case, reported symptoms and a positive COVID-19 self-test result, requiring immediate physician notification for risk management. Although confirmation of the COVID-19 diagnosis would have been ideal, additional testing could not be determined because the donor was lost to follow-up. However, despite that COVID-19 self-tests are not as sensitive as PCR-based tests, it is very likely that the patient was infected with COVID-19.[15,16]

At the time of this writing, no donor screening test for COVID-19 is required by the FDA. Donors are currently screened for symptoms of illness before donation, including answering the first question on the donor history questionnaire, "Feeling healthy and well today?", in addition to a temperature, blood pressure, and pulse rate assessment during the donor physical examination.[17] Donors who are feeling unwell or exhibiting signs and symptoms of illness, including respiratory illnesses, are deferred from donation.

The SARS-CoV-2 virus, which is the virus implicated in COVID-19, has been known to cause viremia in those infected with COVID-19, raising the concern for transfusion-transmitted COVID-19. However, respiratory viruses in general have not been shown to be transmitted through blood transfusion. Nevertheless, SARS-CoV-2 RNA has been detected in the plasma of blood donors who reported a diagnosis of COVID-19 after donation, albeit in a small fraction of donors and at very low levels.[18,19] The current body of scientific evidence reveals rare case reports that demonstrate no transmission of COVID-19 through transfusion of blood products from donors who report PDI of COVID-19 infection.[20-23] Specifically focusing on granulocyte transfusion, there have been 2 previously reported instances of granulocyte transfusion to patients from donors infected with COVID-19; both demonstrated no evidence of transmission of COVID-19 via transfusion.[22]

In summary, the case demonstrates several unique aspects of transfusion medicine, including clinical management of PDI and risk assessment when a patient is transfused with a blood product from a donor with an active COVID-19 infection. In the circumstance of our case individual, she did not develop new COVID-19 symptoms and tested negative for COVID-19 after transfusion of the implicated granulocyte unit. This finding adds to the body of scientific evidence that COVID-19 is not transmitted via blood transfusion.

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Conflict of Interest Disclosure

The authors have nothing to disclose.

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Uncommon causes of hemoglobin E flags identified during measurement of hemoglobin A1c by ion-exchange high-performance liquid chromatography

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Abbreviations: Hb, hemoglobin; HPLC, high-performance liquid chromatography

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ABSTRACT

We present 3 cases of discordant results from screening hemoglobin A1c (HbA1c) measured by ion-exchange high-performance liquid chromatography (HPLC) all due to various forms of interference and flagged by the instrument as "suspected hemoglobin E (HbE)." The first case was due to a rare hemoglobin variant, later confirmed to be hemoglobin Hoshida, the second due to "true" heterozygous HbE, and the third a result of analytical artifact causing splitting of the HbA1c peak without an underlying variant hemoglobin. We examine the similarities in these cases along with the laboratory work-up to classify each cause of interference to demonstrate the wide array of potential causes for the suspected HbE flag and why it warrants proper work-up. Because there is no standardized method of reporting out hemoglobin variant interference in HbA1c measurement, we discuss our laboratory's process of investigating discordant HbA1c measurements and reporting results in cases with variant interference as 1 possible model to follow, along with discussing the associated laboratory, ethical, and clinical considerations. We also examine the structure of hemoglobin Hoshida, HbE, and conduct a brief literature review of previous reports.

Case 1

The first case involved a 58-year-old man with a medical history of essential hypertension, obstructive sleep apnea, and hyperlipidemia who had a screening hemoglobin A1c (HbA1c) measurement ordered by his primary care provider (**TABLE 1**). He did not have a previous diagnosis of diabetes mellitus and had 2 normal previous HbA1c measurements (4.4% and 4.7%) but demonstrated borderline elevated fasting glucose levels (most recently 105 mg/dL). His HbA1c was first measured using our primary HbA1c testing method, an ion-exchange high-performance liquid chromatography (HPLC; Tosoh G8, v5.23F) with the result of 4.4%. An unknown peak with a retention time of 0.73 minutes (1.6% area) was flagged as suspected interference by what the instrument software identified as possible HbE (**FIGURE 1A**). Based on the flag, additional investigation included testing the specimen using an alternative in-house method for HbA1c measurement, a turbidimetric inhibition immunoassay (Roche cobas c502), which resulted as 5.7%. The difference between the HbA1c results obtained by HPLC and immunoassay raised the possibility of a hemoglobin variant that interfered with the HPLC method, and further testing was undertaken to characterize the variant.

In this first case, the variant hemoglobin coeluted with HbA2 on the VARIANT II HPLC at 3.60 minutes, with the peak accounting for ~45% of total hemoglobin in the sample (**FIGURE 2A**). The observed retention time is within the range of when HbE typically elutes (3.59-3.74 minutes, midpoint 3.67 minutes). However, the percentage of HbE typically ranges from 24% to 29% in patients with heterozygous HbE, 87%-88% in patients with homozygous HbE, and between 30%-70% in patients with HbE β thalassemia, depending on the severity of the thalassemia.[1,2] By capillary electrophoresis (CE), the variant migrated at the upper limit of the HbF zone, accounting again for approximately 45% of the total hemoglobin (**FIGURE 2B**). The combined results obtained from VARIANT II HPLC and CAPILLARYS CE allowed us to narrow down the list of suspected heterozygous beta chain variant hemoglobin, but definitive classification required additional testing. The ordering physician was contacted to discuss the case, and additional confirmatory testing was requested following a discussion with the patient. As such, separate hemoglobin variant analyses performed at a reference laboratory included beta globin gene sequencing, which revealed the presence of GAG to CAG mutation at codon 43, consistent with hemoglobin Hoshida.

Case 2

The second case involved a 14-year-old healthy transgender female being followed by pediatric endocrinology for hormone replacement therapy who underwent a screening HbA1c with a fasting glucose of 87 mg/dL.

TABLE 1. Complete blood count and glycemic status laboratory results for three patients flagged with suspected hemoglobin E (HbE)

	Patient 1 (with suspected HbE)	Patient 2 (with suspected HbE)	Patient 3 (with suspected HbE)
Age, y	57	14	36
Sex	M	M	F
Red blood cells (reference range), x 10^6/μL	4.47 (4.40-5.70)	5.36 (4.50-5.50)	4.25 (3.90-5.00)
Hemoglobin (reference range), g/dL	14.5 (13.5-17.0)	13.5 (12.5-16.0)	12.5 (12.0-16.0)
Hematocrit (reference range), %	40.7 (40.0-50.0)	41.8 (36.0-49.0)	37.6 (36.0-48.0)
Mean corpuscular hemoglobin (reference range), pg	32.4 (27.0-32.0)	25.2 (24.0-35.0)	29.4 (27.0-32.0)
Glucose (reference range), g/dL	105 (73-100)	87 (73-100)	Not available
HbA1c (Tosoh G8), %	4.4	4.7	3.7
HbA1c (Roche c502), %	5.7	5.7	4.8

The patient's sample tested by Tosoh G8 HPLC showed an HbA1c of 4.7%, with an unknown peak at retention time 0.68 minutes (1.6% area) that was flagged by the software as suspected HbE, similar to the first case (**FIGURE 1B**). Analysis of the patient's sample by immunoassay resulted in an HbA1c value of 5.7%.

In this case, the hemoglobin variant similarly coeluted with the HbA2 (retention time 3.72 minutes) and accounted for 26% of total hemoglobin, a pattern consistent with heterozygous HbE (**FIGURE 2C**). The presence of HbE was confirmed by CE (**FIGURE 2D**).

Case 3

The third case involved a 36-year-old healthy female who had a screening HbA1c ordered as part of a routine health maintenance visit. Tosoh G8 HPLC measured HbA1c as 3.7% (below reportable range) with an unknown peak at retention time 0.65 minutes (1.1% area), again flagged as suspected HbE. By immunoassay, HbA1c was 4.8%, and the specimen was sent for further work-up, similar to the above cases.

In contrast to the first 2 cases, the third case had unremarkable VARIANT II HPLC and CE chromatograms without any hemoglobin variant identified (**FIGURE 2E** and 2**F**). After consideration of this finding, a careful reevaluation of the Tosoh G8 HPLC chromatogram of case 3 identified that the P-HV3 peak flagged by the instrument was likely due to an exceedingly rarely observed peak splitting and integration problem (**FIGURE 1C**). In support of this observation, the sum of SA1c and P-HV3 peak areas corresponded to the 4.8% HbA1c value obtained by immunoassay.

Of note, all 3 patients had no history of hemolysis and concurrent blood work (basic metabolic panel) did not suggest hemolytic processes causing falsely low HbA1c. All 3 samples underwent independent testing on VARIANT II ion exchange HPLC (Bio-Rad—Beta-thalassemia short program) and CAPILLARYS (Sebia—HEMOGLOBIN(E) assay) CE (**FIGURE 2**).

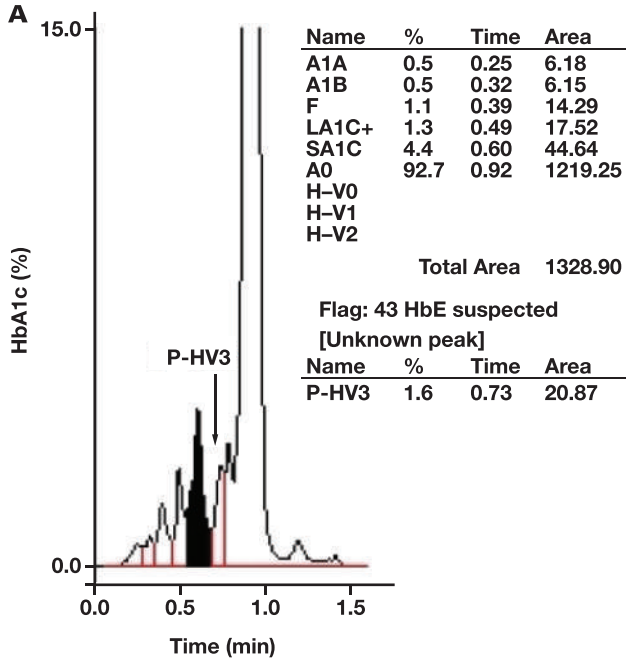
Discussion

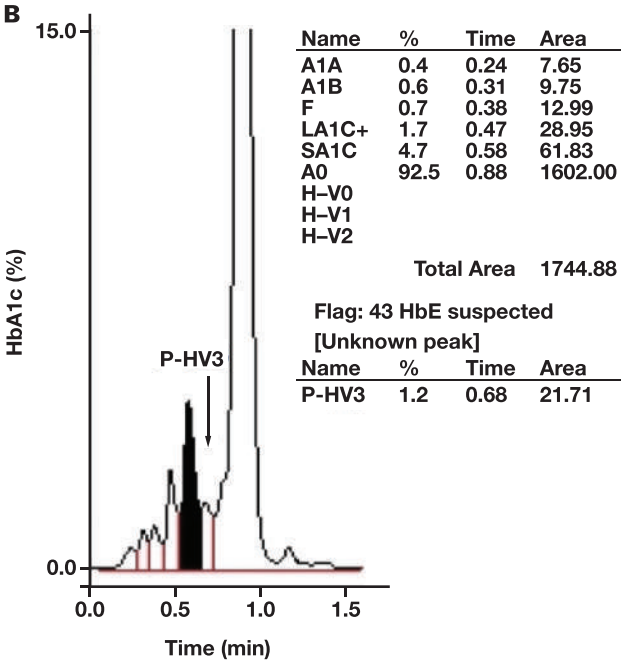
Hemoglobin is a tetrameric protein with 4 subunits each comprised of 1 unit of heme, an organic porphyrin ring that binds inorganic iron, and 1 unit of globin, a polypeptide chain.[3] The majority of adult globin chains are either alpha or beta chains, with a few gamma or delta chains. The alpha chain is encoded by 2 genes on chromosome 16 (HBA1 and HBA2) and is 141 amino acids in length, whereas the beta chain is encoded by the HBB gene on chromosome 11 and is 146 amino acids in length.[4] Combinations of these globin chains create Hb A ($\alpha 2\beta 2$), A2 ($\alpha 2\delta 2$) and F ($\alpha 2\gamma 2$). Glycated hemoglobin, or more commonly referred to as HbA1c, is formed in the presence of excess glucose in the bloodstream through a nonenzymatic process between glucose and the N-terminus of the beta globin chain.[5] This reaction takes place in 2 steps: first with the condensation of blood glucose onto select amino acid residues creating the "labile" HbA1c, which reflects acute blood glucose concentrations, and second with an irreversible Amadori rearrangement to a stable ketoamine.[6] Once the 2-step reaction has occurred, the hemoglobin will remain glycated throughout the erythrocyte's life span, which is approximately 3 months.[7] These observations have caused HbA1c measurement to be used as a popular screening and monitoring test for diabetes.

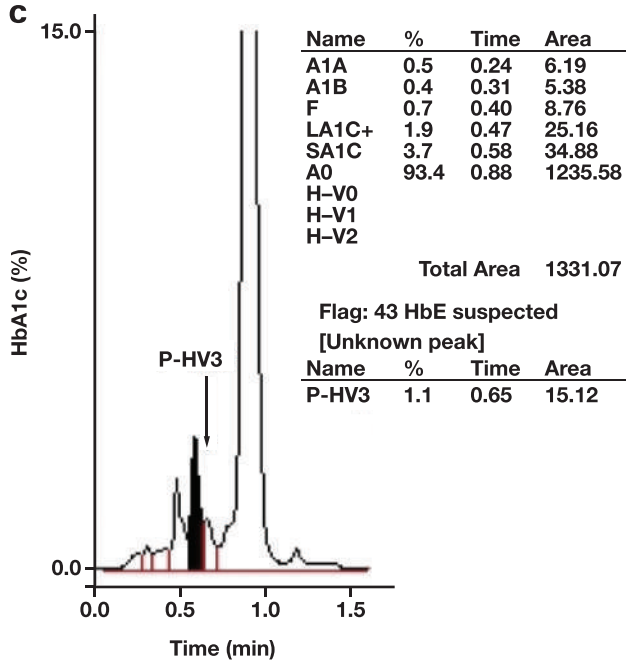
There are various methods to determine HbA1c, including separation methods such as ion-exchange HPLC, boronate affinity HPLC, and CE, and chemical methods such as enzymatic assays and immunoassays.[8] Interference from Hb variants can affect the measurement abilities of these methods to varying degrees. With ion-exchange HPLC, for instance, there are multiple methods of interference that variant hemoglobin chains could have on A1c measurement due to either biologic or analytical causes. Biologic causes include pathogenic mutations that lead to structural changes that alter the glycation process and subsequent HbA1c formation, which could affect multiple measurement methods. One such example is due to substitutions of amino acids at glycation sites that alter the rate of glycation of hemoglobin, seen in Hb Raleigh.[8] Alternatively, some pathologic Hb variants can affect red cell turnover and decrease erythrocyte lifespan affecting the measurement of HbA1c.[9] In patients with altered erythrocyte life span either caused by pathologic Hb variants or other conditions (pregnancy, HIV, hemodialysis, recent blood loss or transfusion) that preclude accurate reporting of HbA1c measurement in all available methods, measurement of alternate glycated proteins may have to be implemented. Such alternative tests include measurement of fructosamine and/or glycated albumin. However, it should be mentioned that the American Diabetes Association suggests that the measurement of these analytes does not translate as accurately with average blood glucose levels as does HbA1c, mostly due to their shorter half-lives of about 2 weeks compared with 2-3 months for HbA1c.[9,10]

The most common interferences are Hb variants, elevated levels of HbF, and derivatives that affect the analytical accuracy of a HbA1c result. An example of an analytical interference with HPLC occurs due to mutations that change the charge or configuration of hemoglobin and

FIGURE 1. **Tosoh G8 high-performance liquid chromatograms for patient samples flagged as suspected hemoglobin (Hb)E. A, Case 1 with hemoglobin Hoshida. B, Case 2 with HbE. C, Case 3 with analytical artifact of split HBA1c peak and no underlying hemoglobin variant. SA1c: HbA1c.**

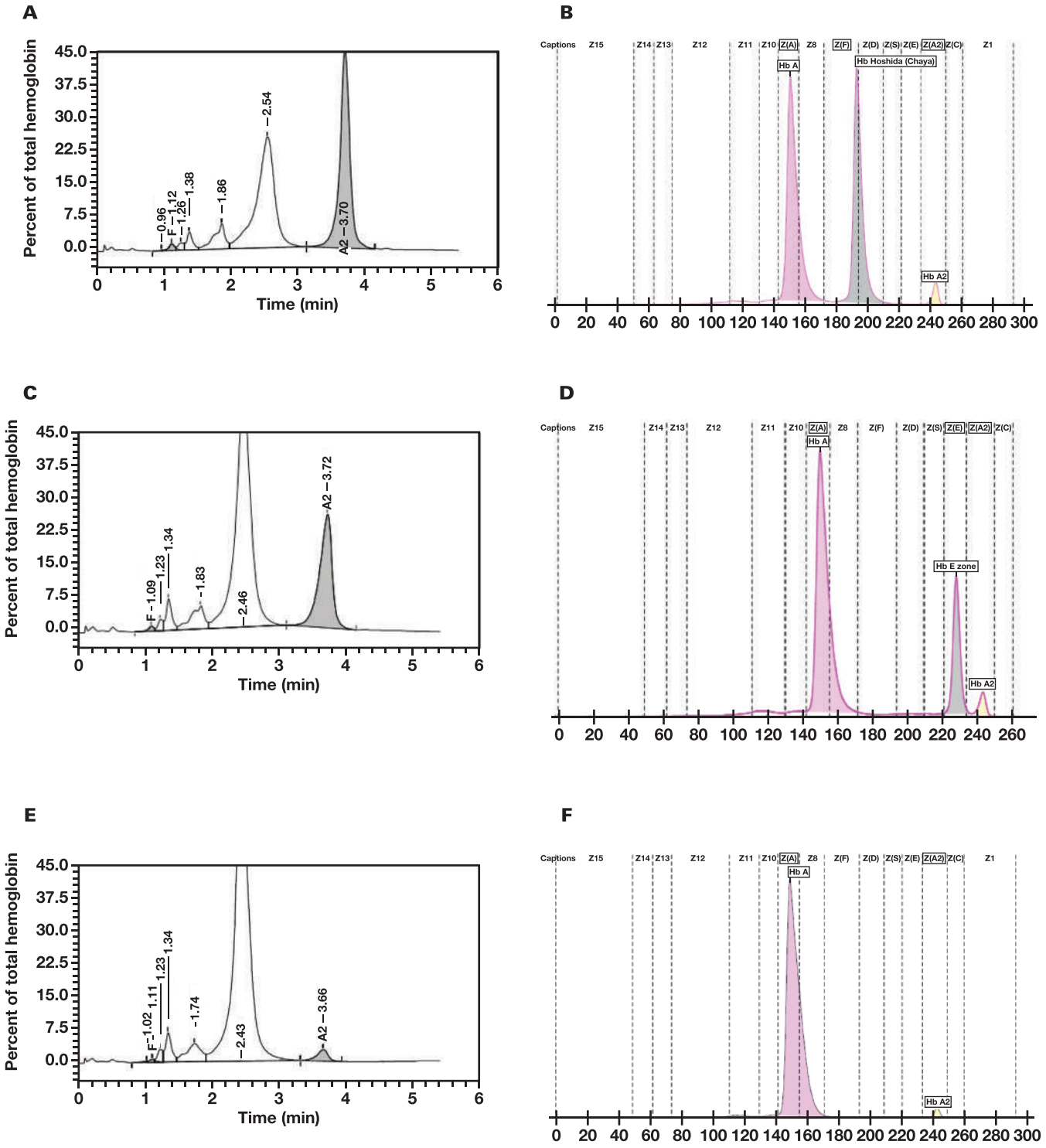






can coelute with the peaks of interest and interfere with HbA1c measurement; however, the chromatogram read-out of the analyte interference can frequently be used to identify common variants that require additional confirmation testing.[11] First-generation immunoassays were affected by analytic interference from common Hb variants, notably HbS and HbC, due to similarities in the epitope recognized by these antibodies. Compared with first-generation immunoassays and HPLC, newer-generation immunoassays are less prone to interference from common Hb variants due to their increased analytic specificity for the N-terminal of the β-globin chain of HbA. However, these newer assays still have limitations in that they do not have the ability to identify when a variant hemoglobin is present in the sample and can have the potential to give erroneous HbA1c results in patients lacking HbA.[11,12] In contrast, ion-exchange HPLC can potentially identify when a hemoglobin variant is present, which maintains its reputation as a reference method and the gold standard for HbA1c measurement. Immunoassays

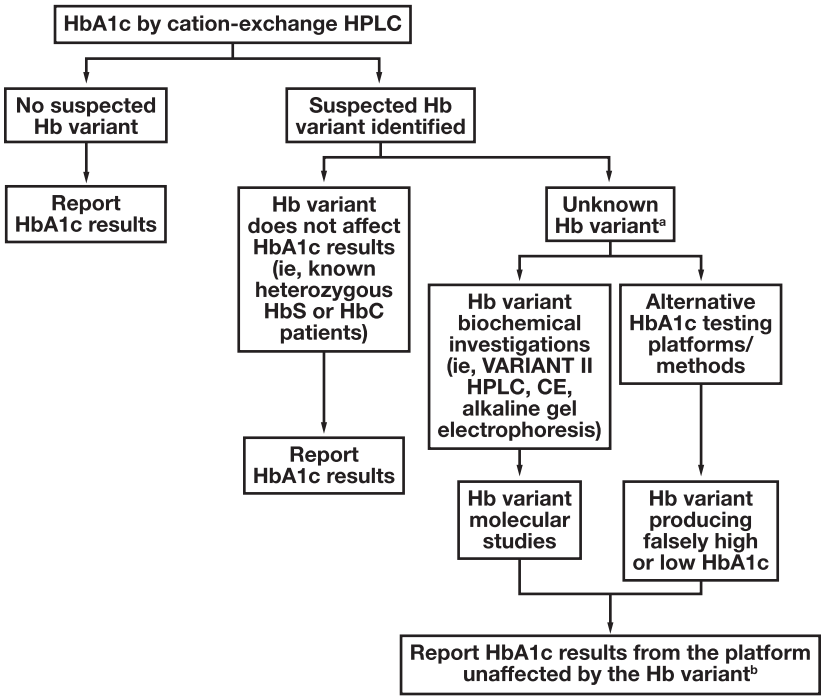
FIGURE 2. High-performance liquid chromatography (HPLC) and capillary electrophoresis for patients flagged with suspected hemoglobin (Hb)E using Tosoh G8 HPLC for HbA1c measurement. A, B, Hb Hoshida. C, D, HbE. E, F, No Hb variant/split HbA1c peak.



can be cheaper in cost and produce faster results; however, they tend to have lower precision than HPLC methods.[13] According to a 2014 College of American Pathologists (CAP) survey, HbA1c measurement by immunoassay had a coefficient of variation ranging from 1.6% to 6.1%, compared with 1.6% to 2.7% for ion-exchange HPLC and 2.1% to 3.1% for boronate affinity HPLC.[14] More recent CAP surveys support the differences seen, albeit smaller, in performance, with coefficient of variation ranges of 1.0%-3.9% for immunoassay and 0.8%-2.5% for ion-exchange HPLC.

It should be noted that depending on the resolution of the HPLC instrument being used, certain Hb variants can coelute near common variant peaks, creating the potential for the interfering hemoglobin variant

FIGURE 3. Potential algorithm for first-time investigation of hemoglobin (Hb) variants initially identified by HbA1c ion exchange–high-performance liquid chromatography (HPLC) testing. [a]Laboratories may choose to report the HbA1c results as a comment: "Unable to quantitate HbA1c by this method due to interference (by a presumptive Hb variant)" or perform additional laboratory investigations depending on resources and preferences. [b]Reports can include the HbA1c results and a comment indicating interference with capillary electrophoresis (CE) or HPLC and method used for reporting.



to be misinterpreted. Although coelution was not a confounding factor in the cases reported here, the presence of the uncommon variants and the analytical artifact in distinct specimens were flagged by the software with the same presumptive HbE variant, and further work-up was needed to correctly identify the variants and assess which HbA1c method should be used for reporting. These 3 cases exemplify how seemingly different causes of interference can create a common shared flag within the instrumentation software and why it is vitally important to fully investigate these flags to discover the root cause. Of note, a newer version of the Tosoh G8 HPLC software (v5.24F) replaces the "Hb E Suspected" flag with "Check Peaks" for chromatograms exhibiting a P-HV3 peak between SA1c and HbA0, and HbA1c results can be reported in the presence of HbE per the manufacturer notifications. However, despite this claim, confirmation testing may still be needed for first evaluation of patients with the HbE trait, as it is uncertain from the A1c chromatogram what the underlying cause of the flag may be. Even so, it is notable that there is disparity in HBA1c values between methods in our case with heterozygous HbE.

The process of reporting incidentally identified Hb variants is not standardized and practices vary between laboratories. A general algorithm for investigating HbA1c results with suspected Hb variants is shown in **FIGURE 3**. In all 3 cases, patient samples were tested using an alternative HbA1c testing method. However, a different extent of investigation for Hb variants was warranted in each of the 3 cases above (and as reflected in our institution's investigative algorithm). Complex cases such as Hb Hoshida require extensive investigations that may include VARIANT II HPLC, CE, and confirmatory molecular studies to fully characterize the interfering variant. In other cases, such as HbE, the presumptive Hb variant may be confirmed using VARIANT II HPLC and/or CE only. Finally, for the case of artificial splitting of the HbA1c peak, VARIANT II HPLC essentially ruled out the presence of a variant.

Ethical considerations regarding the reporting of Hb variants relate to the potential for patients to undergo reproductive counseling as possible carriers, as well as their access to health information that may have direct health impacts (eg, the increased risk of chronic kidney disease seen in sickle trait). Given that most incidentally found Hb variants are clinically benign, there may be a negligible risk in reporting them. Despite this, many clinical laboratories choose not to report variants unless a variant evaluation is ordered; alternatively, laboratories may report the presence of a presumptive Hb variant, or more generally as an interference, only if they interfere with HbA1c measurement.[15]

Hemoglobin Hoshida, highlighted in our first case, is a beta chain variant in which a GAG to CAG mutation in codon 43 causes a change from glutamic acid to glutamine. Its name is derived from the village in Japan in which it was first identified in a family. It is known to be a stable hemoglobin variant with normal function that ranges in proportion from 42% to 45% in heterozygous individuals.[16] Although there have only been rare reports of this hemoglobin variant,[16-18] it has been shown to interfere with HbA1c measurement using HPLC methods and cause a lower result, consistent with what was observed in this particular case.[15] Hemoglobin Hoshida does not interfere with immunoassay methods for HbA1c, as the mutation is located away from the N-terminal β-globin chain epitope used for HbA1c measurements, supporting the use of immunoassay methods for reporting HbA1c for patients with Hb Hoshida.

In contrast to Hb Hoshida, HbE is a relatively common B-globin variant due to a substitution of glutamic acid by lysine at codon 26 that

occurs in high frequencies in certain Asian countries.[2] Hemoglobin E syndromes can range from asymptomatic to severely symptomatic, with the most severe form resulting from HbE/B^0 thalassemia.[19] Like Hb Hoshida, HbE is known to cause interference in HPLC measurement of HbA1c leading to falsely low measurements, as seen in our second case discussed.[20]

Conflict of Interest Disclosure

The authors have nothing to disclose.

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IN MEMORIAM

Robert L. Schmidt, MD, PhD, MBA (November 17, 1952-October 25, 2023)

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Robert Lane Schmidt, MD, PhD, MBA, was a clinical pathologist, scholar, and educator with wide-ranging expertise in numerous subjects. His recent work at LabCorp Health Systems centered around population health informatics/analytics, operations analysis, quality improvement, and laboratory stewardship. Dr Schmidt was previously a professor in the Department of Pathology at the University of Utah School of Medicine and founding director of the Center for Effective Medical Testing at ARUP Laboratories. Prior to that, he was a professor at Marshall School of Business at the University of Southern California. Dr Schmidt earned numerous undergraduate and graduate-level degrees in medicine, data science, informatics and biostatistics, science and engineering, and business. Dr Schmidt was a model lifelong learner.

Dr Schmidt's proudest academic accomplishments were his teaching experiences and coauthoring publications with a remarkably diverse group of collaborators. He designed over 70 education courses in 10 different subject areas and delivered over 2000 lectures throughout his career. Dr Schmidt was recognized as the top instructor in *The Business Week Guide to The Top 40 Business Schools* in 1995, and he earned first place outstanding teaching awards for 2 consecutive years at the University of Minnesota. He served as an editor for *Laboratory Medicine* from 2012 to 2021. Over the course of his career, he reviewed manuscripts for 41 journals and authored nearly 200 publications. He curated an impressive personal home library of academic texts and references and was a true academician.

Helping others grow their careers was the most meaningful aspect of Dr Schmidt's faculty roles. His collaborators benefited from the scholarly productivity that he consistently maintained. Colleagues would consult Dr Schmidt on difficult research questions, often involving statistical techniques and complex data analysis. He was particularly adept at applying concepts learned in one field/area of expertise to a challenging problem in another.

Over the course of his life, Bob lived on multiple continents and loved to travel. He embraced a love of the outdoors and was an expert backpacker and kayaker. In his later years, he lived in the mountains outside of Salt Lake City, where he found great joy in hiking, playing classical guitar, and spending time with his beloved wife, Marianne, and their dogs. Dr Schmidt was a unique person who will be remembered for his humble nature and generous spirit. The memory of his storytelling, sense of humor, and infectious laugh will always be cherished by the family, friends, and colleagues he left behind.