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The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

In this issue

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Foreword from the editor-in-chief

János Kappelmayer, MD, PhD

This recent issue of the eJIFCC contains four additional manuscripts on topics that were presented at the Rome POCT Conference held between September 6-7, 2021. Event information: https://www.ifcc.org/media/478963/poct_roma_2021_preliminary_3nov2020_132641875465705822.pdf.

The organization of this conference began in the midst of the pandemic, when colleagues around the world were under tremendous pressure to fight Covid-19. The meeting had been postponed to a new date and finally it was held successfully in the form a hybrid conference with almost 200 registrations from 37 different countries (122 in person and 76 online).

In particular, the role of POCT was also highlighted during the Covid-19 pandemic in screening and monitoring programs. The aim of this conference had been to bring together IFCC and EFLM experts and representatives of IVD companies, in order to discuss the various dimensions of the POCT: Quality Assurance, Training, Technological Innovations, Applications, Market and Sustainability.

An impressive amount of information was delivered on the Quality issues concerning POCT in the form of quite a few presentations, an article on one of these talks is featured in this issue authored by Julie Shaw. Another session focused on training for POCT, and the presentations like that of Scott Isbell in this Issue outlined that a proper training and competency assessment is mandatory to ensure that test results are accurate and reliable, and furthermore, highlighted the difference between the assessment-based certificate program and the professional certification at the AACC. The experience of the AACC could also be a model for other National societies and/or for supranational Clinical Laboratory Federations. An additional two manuscripts from this meeting are also included in this eJIFCC issue. The recent pandemic highlighted the desire for Public Health Education on POCT for both Health professionals and end-users as reported by Gerald Kost, while Tommaso Trenti addresses the issue of possibility of synergy between the consolidation processes of Laboratory Medicine, central Laboratories and POCT. The answer is definitely a yes, where effort and input from all clinical laboratory professionals are required.

I think we should be grateful for the perseverance of the organizers, particularly Professor Sergio Bernardini, and all the participants, particularly to those who undertook the travel burdens in these uneasy times and made this IFCC/EFLM conference a lively event.

János Kappelmayer
Editor-in-chief, eJIFCC

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Identifying and reducing errors in point-of-care testing

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POCT, quality assurance, audits, quality improvement

ABSTRACT

Point-of-care testing (POCT) refers to diagnostic testing performed outside of the central laboratory, near to the patient and often at the patient bedside. This testing is generally performed by clinical staff who are not laboratory trained and, as such, often do not appreciate the importance of quality assurance (QA) activities aimed at ensuring the quality of testing performed. Within hospital environments, it is typically the central laboratory that oversees POCT and that ensures QA practices are in-place. Audits for compliance of POCT users with policies and procedures in place are key to informing quality improvement initiatives. Here, audit and follow-up data and the results from three quality improvement initiatives are discussed. These examples demonstrate where QA audit practices led to a reduction in POCT errors and improved the quality of result interpretation.

INTRODUCTION

Point-of-care testing (POCT), as defined by the International Federation of Clinical Chemistry (IFCC) POCT working group is diagnostic testing at or near the site of the patient [1]. POCT is performed outside the central laboratory and is most often performed by clinical staff rather than laboratory trained staff [2].

Quality assurance practices are key to ensuring that POCT results are accurate and reliable. This is especially important given that POCT results are immediately available to clinicians for action, with no prior review by a laboratory technologist, as would be the case with central laboratory reported results. International guidelines for POCT and local Laboratory Accreditation standards include many quality assurance practices that must be adhered to [3-5]. Performance of regular audits for compliance of POCT operators with policies and procedures is an important component of quality assurance [4-5]. Audit data reveals areas for improvement and provide a useful starting point for further investigation, follow-up and education. At The Ottawa Hospital, audits are performed annually, at minimum for each POCT program.

Here, data and follow-up from QA three audits performed at The Ottawa Hospital are discussed. Positive patient identification, Charting of POCT glucose results and Inter-instrument comparisons. These examples demonstrate the importance of various QA practices in place for POCT in improving quality and reducing errors in POCT.

METHODS

Patient data for POCT

POCT glucose patient results were obtained from Cobas IT 1000 POCT data management software (Roche, Laval QC). Prior to October 2017, activated clotting time (ACT) patient results were retrieved manually from the Medtronic ACT plus instruments for analysis. In October 2017, the ACT instruments were connected to Cobas IT 1000 POCT data management software and patient results were obtained from Cobas IT 1000 for analysis.

Patient chart audits

Chart audits were performed using the hospital electronic medical record (EMR). These studies were deemed to be quality assurance and did not require research ethics board (REB) approval.

Inter-instrument comparisons

Glucose measurements by the Roche Accuchek Inform II glucose meters used for POCT are regularly compared to glucose measurements by the central laboratory chemistry instrument (Siemens Vista) and central laboratory blood gas analyzer (Radiometer ABL90). Comparisons are made using whole blood (ABL90 and glucose meter) and plasma from the same specimen (chemistry analyzer). A sub-set of glucose meters (n=20) undergo inter-instrument comparison each month.

Inter-instrument comparisons for all blood gas instruments in use, both in the central laboratory and for POCT, are performed monthly using whole blood specimens.

RESULTS

Positive patient identification

In 2016, an audit of the Activated clotting time (ACT) program was completed for testing performed in the operating room. Of the 306 results audited, 141 (46%) were documented in the Anesthesia record in the patient chart. A detailed chart audit indicated that 55 of the 306 results may have been tested using an incorrect patient medical record number (MRN). The chart audit found that these tests were performed

outside the date and time the patient was in the operating room, according to the case notes. Follow-up with the clinical area revealed that clinical staff performing POCT were not entering the patient MRN before performing each test. An investigation by the POCT team found that the instruments were programmed such that they did not require entry of a patient MRN prior to each test. This practice had been in place for a long time as a convenience to the clinical staff, given the large number of tests performed during cases in these Operating Rooms (ORs).

These audit findings raised concerns at a time as there was an ongoing initiative to connect the ACT instruments to the laboratory information system (LIS) and EMR. This could have resulted in patient results transmitting to an incorrect patient chart and this implementation had to be delayed given the risk. The POCT team worked closely with leaders in the clinical area to educate all clinical operators performing ACT testing of the importance of entering a valid patient MRN prior to each test. Barcode scanners were installed for each instrument to make it easier for clinical staff to enter the MRN quickly and accurately. A two-hour time-out feature was activated on the ACT instruments so that the instruments required entry of a patient MRN more frequently, lowering the likelihood of tests being performed under the wrong patient MRN. A follow-up audit in 2017 found that only 5/199 (2.5%) of results were suspected to have been performed under the incorrect patient, indicating that the barcode scanners and time-out feature were effective. Prior to the ACT instruments being connected to transmit results to the LIS and EMR, the instrument settings were configured so that a patient MRN must be entered prior to each test performed.

Documentation of POCT glucose results

Up until late in 2014, POCT glucose results were charted manually using paper-based patient medical records, which were scanned into the patient EMR at The Ottawa Hospital. An audit from February 2014 analyzed compliance with documentation of POCT glucose results in patient charts by clinical staff in the Emergency Departments. Of the 106 results audited, 48 (45%) were found documented in the patient chart and only 31 (29%) of those were specified as POCT results. None of the results were associated with a reference interval or units of measurement. In September 2014, POCT glucose meters at The Ottawa Hospital were interfaced to the Laboratory Information System and EMR via POCT data management software. A follow-up audit in February 2015 for the Emergency Department found that 93% of results were documented in the patient electronic medical record and were documented with appropriate reference intervals. Those results that were not documented were from instances where an "unregistered patient identification number" was used for testing. These numbers are available to the Emergency Department for urgent testing required at Triage prior to the patient being registered. Results are manually documented in the patient EMR once the patient is registered.

Inter-instrument comparisons

At the Ottawa Hospital, we perform regular comparisons between POCT instruments and central laboratory instruments that measure the same analyte. Data from these analyses revealed a small positive bias (0.3-0.4 mmol/L) for glucose measurements below 3.0 mmol/L for the glucose meters in our institution compared to the central laboratory. This information was invaluable when I was contacted by the Nurses in Neonatology regarding what they considered clinically significant differences between POCT and central laboratory glucose measurement in patients being investigated for hypoglycemia. The comparison data available from our regular

inter-instrument comparisons was used to modify the algorithm being used by Neonatology to guide treatment of neonatal hypoglycemia. These findings demonstrate the importance of understanding any bias that exists between POCT and central laboratory instruments.

In another example, during a regular comparison between the central laboratory blood gas instrument and the instruments used for POCT in the OR, one instrument was noted to have discordant pCO_2 values compared to the other POCT and central laboratory instruments. This finding prompted removal of the instrument from clinical service for investigation. Review of the internal QC data from the instrument found that the pCO_2 QC had failed during several measurements but the instrument still provided pCO_2 results to the operator. The central laboratory instruments are configured to repress results for any analytes with QC failures. Further investigation of the POCT instrument in question revealed that it was not configured exactly as the central laboratory instruments. This was corrected. Several patients had inaccurate pCO_2 results reported during the time frame the instrument remained in service with QC failures. The attending physicians for all of these patients were made aware of the issue and, fortunately, there were no negative outcomes.

DISCUSSION AND CONCLUSIONS

Following implementation of POCT glucose results transmission to the EMR, feedback received from physicians was - how much easier it was to find POCT glucose results in the patient chart - which offered a huge benefit in them being able to access the electronic results from home when on-call. In a 2001 study, Kost et al. [4] surveyed forty-six experts in POCT on how medical errors can be prevented related to POCT. The consensus from the survey was that bidirectional connectivity capability was key.

The use of POCT data management software also provides a platform for analysis of patient data related to quality initiatives. Tighter glycemic control in hospitalized patients is recognized as important for improved outcomes and the availability of electronic patient blood glucose results from POCT can be used to analyze success of initiatives aimed at improving glycemic control [5].

Physicians will use POCT results and central laboratory results for the same analyte interchangeably, making it imperative for physicians to be aware of differences between these results that could be clinically significant [6-7]. This has been demonstrated by other studies [8-9] as well as in the current study, highlighting the importance of regular comparison studies between POCT and central laboratory instruments.

Quality assurance practices for POCT, overseen by laboratory professionals are key to ensuring high quality POCT programs and results. The examples of quality assurance described here demonstrate the importance of proper oversight of POCT programs, which is relatively well-defined and in-place in hospital environments. As POCT moves outside of regulated hospital environments, consideration must be given to how these programs will be managed and overseen to ensure appropriate quality assurance practices are in place.

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In this issue: Focusing on POCT

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Professional certification in point-of-care testing

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ABSTRACT

Professional certification is affirmation and documentation that the certified individual has the knowledge, training, and skills necessary to practice some aspect of medicine or other profession. Herein is a description of the genesis of a professional certification in point of care testing (POCT), inclusive of rationale and goals. A distinction between professional certification and certificate training programs is made. Details regarding eligibility to sit for the board exam are provided along with a list exam content areas. Finally, successes of this professional certification program are highlighted.

INTRODUCTION

Per recent market reports the size of the point-of-care testing (POCT) sector is anticipated to reach USD 50.6 billion by 2025 up from USD 29.5 billion in 2020 (1). Demand remains high for easy to use, portable technologies providing rapid results, that when properly integrated into clinical workflows, have been shown to improve patient outcomes, increase healthcare provider and patient satisfaction and decrease cost (2). Point of care testing can be found throughout the hospital – in operating rooms, the emergency department, intensive care units, and the general medical wards. POCT devices have proven useful in the field as tools to assess patients during transport to hospital within ambulance and helicopters. They are found in long term care facilities and are often used to make transfer of care decisions. The personnel operating these devices usually are not laboratory medicine professionals trained in clinical laboratory science but rather other healthcare professionals such as nurses, respiratory therapists, and medical assistants. In the U.S.A., pharmacists can be added to this list, given the expansion of POCT in community/retail pharmacy settings. Per the U.S.A based National Community Pharmacists Association (NCPA),

> *Point-of-care testing provides an excellent opportunity for community pharmacies to enhance revenue by expanding patient care services while improving health at the patient and population levels (3).*

Community pharmacies offer an attractive alternative to the emergency department for minor acute illnesses, especially given their abundant locations and expanded hours. For more information of one example, I point you to a recent review in the *Journal of Clinical Microbiology* by S. R. Herbin, D. G. Klepser, and M. E. Klepser on the subject of pharmacy-based infectious disease management programs incorporating CLIA-waived POCTs (4).

POCT TRAINING

Training in the oversight and management of POCT is variable. There is no standardized curriculum to teach principles of best practice in point of care testing embedded within schools of nursing, pharmacy or even medical laboratory technology/clinical laboratory science. Much of training for many POCT personnel occurs on-the-job. Certificate programs in POCT are available to supplement or learn about POCT. These programs include but may not be limited to the AACC POC Specialist Program (elaborated more below), a Pharmacy-based Point-of-Care Testing Certificate Program from the NCPA launched in February 2021, a Point-of-Care Testing (POCT) Specialist REACH Certificate Course from the American Medical Technologists association and the Community Pharmacy-based Point-of-Care Testing Certificate Program from the National Association of Chain Drug Stores (NACD). Based on a review of publicly available course learning objectives the certificate programs offered by laboratory medicine based associations are focused primarily on quality management and the administration of point of care testing programs while pharmacy association certificate programs, having some overlap, appear to be focused more on the clinical application of POCT in a pharmacy setting and include such topics as assessment of clinical signs and symptoms of common infectious diseases (e.g. influenza, group A Streptococcal disease) and the performance of physical assessments (e.g. cervical lymph node examination, oxygen saturation, body temperature).

POC SPECIALIST CERTIFICATE PROGRAM

Recognizing early a gap in POCT education, AACC in collaboration with the Critical and

POCT Division (CPOCT) of the AACC launched in 2008, a Point-of-Care (POC) Specialist Certificate Program - an assessment-based online course, consisting of eight learning modules covering topics including: regulations, quality management, policies/procedures, instrument selection/validation, connectivity/information technology (IT), education/training, administration, and communication (5). Upon completion of the online modules, participants are administered an examination to assess their learning and are provided a certificate of successful completion. To date, 1733 individuals have completed this professional development certificate program. Future versions of this course may be expanded to include international regulatory standards such ISO 15189 (Medical Laboratories) and ISO 22870 (Point of Care Testing) thus providing professional development course with an even broader worldwide application.

ASSESSMENT BASED CERTIFICATE PROGRAMS VS. PROFESSIONAL CERTIFICATION

It is important to mark the distinction between professional certification and a course which yields a certificate. Many professional education courses offer a certificate of attendance: a record that an individual attended the program. A certificate of attendance does not reflect whether an individual has successfully completed the program. Assessment-based certificate programs recognize when a participant has successfully participated in and completed the intended education and training objectives (6). The AACC POC Specialist Program, for example, is an assessment-based certificate program. This educational, professional development course has specific learning outcomes that a participant must prove they have accomplished to be awarded a certificate. The purpose of this program is to provide education and training to be more knowledgeable about POCT applications, knowledge which can then be applied in daily practice. Successful completion is determined by the participant achieving an accomplishment, signified by the provision of an official document, i.e., certificate by an authoritative body, in this case AACC. From an educational perspective, this is akin to certificate programs granted by institutes of higher education (i.e., the authority) where there are requirements and standards (i.e., learning objectives and assessments) that must be satisfied.

Professional certification is the affirmation and documentation that the certified individual has met predetermined standards of knowledge, training, and skills deemed necessary to practice some aspect of medicine or other profession. Professional certifications are administered by a governing body, or board, of relevant stakeholders – often practitioners who themselves have demonstrated an elevated level of learning and competency and are normally certified by the very board they serve on. These boards are legally or administratively autonomous from other entities and responsible for all essential decisions related to the certification activities (6). In the medical field, certifications often become requirements to practice, individuals are said to be “board certified.” Examples of U.S.A. professional certification boards within the specialty of laboratory medicine include but are not limited to, the American Board of Clinical Chemistry (ABCC), the American Board of Pathology (ABP), the American Board of Medical Microbiology (ABMM). American Society for Clinical Pathology (ASCP) Board of Certification, American Medical Technologists (AMT), and the American Board of Medical Genetics and Genomics (ABMGG).

CREATION OF A PROFESSIONAL CERTIFICATION IN POCT

Given the opportunity and position to lead, the continued and predicted growth of POCT, along

with the need to set professional standards, AACC made the establishment of a professional board certification in POCT one of its strategic priorities. In 2017, AACC formed a task force for the development of a professional certification examination to assess one's applied knowledge and competencies in the field of POCT. The task force was populated by individuals with expertise in POCT with a range of educational backgrounds from bachelors to doctoral level (PhD), representing different perspectives (point of care coordinator, medical directors of POCT, and industry scientists). See Table 1. for list of task force members. In addition, the task force relied on the extraordinary work of a larger group of POCT experts to assist with exam development, see Table 2. The task force partnered with professional psychometricians, individuals who, per the U.S.A. based Psychometric Society, are trained in the science of quantitative measurement practices in psychology, education, and the social sciences. In other words, experts in assessments or testing. This partnership was crucial to ensure an accurate and valid assessment was developed. (Table 1 and Table 2)

Following the development of the certification exam an AACC POCT Professional Certification Board was established to oversee all aspects of the professional certification process from application, to examination, scoring, and maintenance of the exam item bank. Two of the members of the task force were selected to serve on the inaugural board along with other well-recognized experts. See Figure 1 for organization and initial population of the board. The goal over time is that the AACC POCT Professional Certification Board will eventually be populated by those individuals that it has certified.

Individuals that successfully meet eligibility requirements and pass the certification exam are credentialed as Certified Point-of-Care Testing Professionals, abbreviated as CPP, and are encouraged to use this credential as a suffix following their educational degree (7). (Figure 1)

Table 1 AACC Professional Certification exam development task force

Name	Title	Institution
T. Scott Isbell PhD, DABCC, FAACC	Medical Director, Clinical Chemistry and POCT	Saint Louis University School of Medicine
Corinne Fantz PhD, DABCC, FAACC	Director, Scientific Affairs POCT	Roche Diagnostic Corporation
Kerstin Halverson, MS	Clinical Applications Manager	Instrumentation Laboratory
Karen Jenkins MT(ASCP)	Point of Care Coordinator	Emory University Hospital – Midtown
Peggy Mann MS, MT(ASCP), CPP	Ambulatory POCC/Program Manager	University of Texas Medical Branch
Elaine Colwell [ex-officio]	Associate Director, Professional Education	American Association for Clinical Chemistry

Table 2 Professional Certification exam item writers

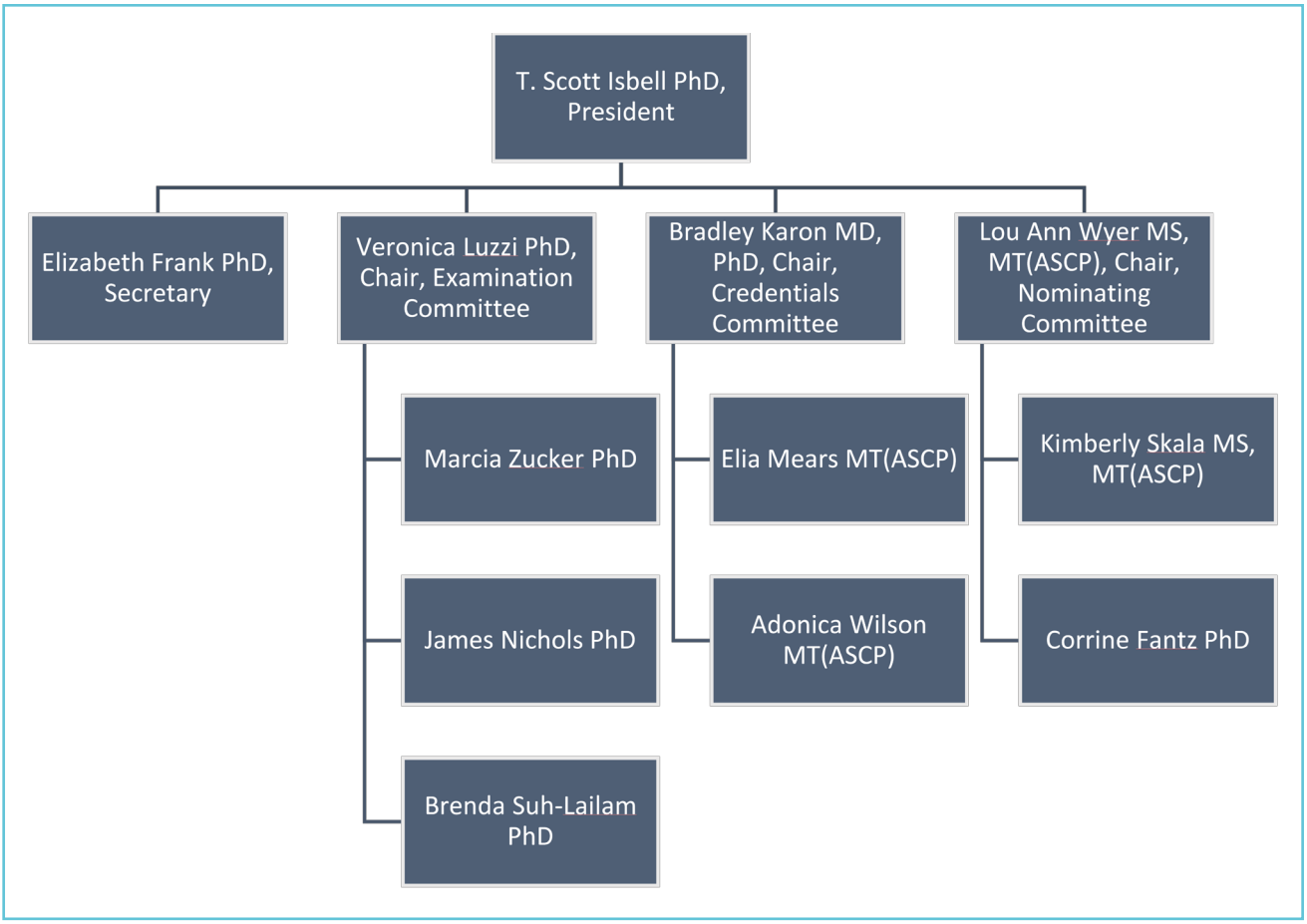
Name	Institution
Adonica Wilson	A. I. duPont Hospital for Children
Alan Wu	University of California at San Francisco
Bob Kaplanis	Laboratory Sciences of Arizona/Banner Health
Bradley Karon	Mayo Clinic
Brenda Suh-Lailam	Ann & Robert H. Lurie Children's Hospital of Chicago
Charbel Abou-Diwan	Nova Biomedical
Charlotte Bismark	Dixie Regional Medical Center
Christine Cursio	University Health Network
Christopher McCudden	The Ottawa Hospital General Campus
Corrine Fantz	Roche Diagnostic Corp.
David Kinninburgh	Alberta Centre for Toxicology
Dawn Smith	UNC Health Care
Elia Mears	Joint Commission on Accreditation of Healthcare Organizations
Elizabeth L. Frank	University of Utah and ARUP Laboratories
Gerald Kost	University of California Davis
James Nichols	Vanderbilt University Medical Center
Julie Shaw	The Ottawa Hospital University of Ottawa and Eastern Ontario Regional Laboratories Association
Karen Jenkins	Emory University Hospital Midtown
Kathleen David	TriCore Reference Lab
Kerstin Halverson	Instrumentation Laboratory

Kim Skala	Instrumentation Laboratory
Lilah Evans	Thomas Jefferson University Hospital
Lou Ann Wyer	Sentara Healthcare
LuAnn Hildebrand	York Hospital
Marcia Zucker	ZIVD LLC
Mark Kellogg	Boston Children's Hospital
Nam Tran	University of California Davis
Nichole Korpi-Steiner	University of North Carolina
Nicole Tolan	Sciex Diagnostics
Octavia Peck-Palmer	University of Pittsburgh Medical Center
Paul Yip	University Health Network
Peggy Mann	University of Texas Medical Branch
Rachel Edwards	Texas Children's Hospital
Robert Nerenz	Dartmouth-Hitchcock Medical Center
T. Scott Isbell	Saint Louis University School of Medicine
Sheila Cruthis	Moses Cone Health System
Sonya Evans	Greenville Health System
Veronica Luzzi	TriCore Research Institute
William Clarke	Johns Hopkins University School of Medicine

The Certified Point-of-Care Testing Professional (CPP) credential certifies individuals as proficient in point-of-care testing.

The credential is evidence that the individual has demonstrated competency in U.S.A. regulations and compliance, quality management, education and training, instrument selection and validation/verification, connectivity and information technology, leadership and communication, specimen types, policies and procedures, clinical applications, and technology and methodology.

Figure 1 Inaugural AACC POCT Professional Certification Board



The first ever professional certification exam in POCT was administered by the board in November 2018. The exam has been administered twice a year in the spring and the fall since 2019. To date sixty-nine individuals have met the requirements of the board, successfully passed the certification exam, and attained CPP certification.

REQUIREMENTS TO SIT FOR THE EXAM

Who is a candidate for POCT professional certification? Professional certification is geared towards those individuals who have significant responsibility related to point-of-care device selection and validation, quality management, operator training, regulatory compliance, and overall management of a point of care program. This could include laboratory managers, nursing managers, point-of-care coordinators, respiratory therapists, pharmacists, pharmacy practitioners, and physician assistants. Professional certification is a visible way to let colleagues, institutions, and your patients know that you are competent practitioner of POCT. While there is a focus on the application of U.S.A. regulatory/compliance knowledge, this does not preclude international applications, all who meet the eligibility criteria will be considered.

To be eligible for the CPP credential, you must have either a four-year degree in a biological, chemical, physical, or medical laboratory science or in nursing and at least two years of direct work experience in point-of-care testing. Alternatively, individuals with a two-year

degree in medical laboratory science or medical laboratory technology and four years of direct work experience in point-of-care testing will be considered. Interested applicants can find eligibility requirements and apply for certification at www.aacc.org/cpp. Applications to sit for the board exam are reviewed by the POCT Professional Certification Board's Credentials Committee. For detailed information please refer to the AACC Point-of-Care Professional Certification – Procedures for Examination and Certification handbook (8).

LOOKING TO THE FUTURE

It is one of the goals of the POCT Professional Certification Board that as the number of individuals credentialed as a CPP grows so does their potential for greater leadership opportunities, greater job secur ity, and higher pay. Testimonials from recently certified individuals are indicating this to be true. Darlene Paskovics, CPP notes, "my CPP allowed me to advance to a leadership position and apply my knowledge to a diverse group of testing platforms," and Christa Williams, CPP states, "achieving my CPP designation has given me an edge in my field and will open new career opportunities." Metrics of success for this professional certification program, will be the observation of increased numbers of CPPs providing technical and quality direction for POCTs, writing practice guidelines, advancing the field through research, and serving on national and international boards and committees concerned with best practice in POCT.

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Public health education should include point-of-care testing: lessons learned from the covid-19 pandemic

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antibody test, antigen assay, coronavirus disease 2019 (COVID-19), curriculum, learning objectives, lessons learned, molecular diagnostics, point-of-care specialists, point-of-care testing (POCT), point of need, public health education, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

Prepared for the eJIFCC with the same title as the presentation by the Speaker in Session 3, Noon, at "POCT: Making the Point," Rome, Italy, September 6-7, 2021.

ABSTRACT

Goal

The goal is to present key principles of point-of-care testing (POCT) in educational curricula that meet critical needs for rapid decision-making in disasters, outbreaks of highly infectious diseases, emergency management, and complex crises.

Observations

The coronavirus disease 19 (COVID-19) pandemic unequivocally proved the value of POC strategies. Striking needs identified by COVID-19 challenges have yet to be entirely fulfilled. A comprehensive national survey showed absence of POCT training in public health colleges, schools, and programs. Fundamental improvements in national structuring of POC knowledge, skills, experience, training, dissemination, accreditation, and licensing are necessary, so that multidisciplinary public health teams can respond effectively and efficiently by geospatially optimizing the control and mitigation of highly infectious diseases and other critical challenges.

Conclusions

Four sets of POCT learning objectives were developed for public health and other educational institutions. Global implementation of POC diagnostics in the hands of trained personnel will help avoid untimely worldwide crises, huge economic losses, uncounted excess mortality, and sudden disruptive surges of dangerous infectious threats to personal security and cultural stability.

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GOAL, BACKGROUND AND METHODS

Goal

To create a long-term public health paradigm of rapid response diagnosis at points of need, this article identifies necessary skills in point-of-care testing (POCT) for new teaching curricula in colleges, schools, and programs of public health **(1,2)**, in order that faculty can educate public health students and practitioners in POC strategies for highly infectious diseases, emergency management, and crisis preparedness**.**

Public health disconnect

The disconnect between current public health practice with testing performed in distant slow reference laboratories versus the demonstrated value of rapid COVID-19 testing in communities directly at points of need may continue to threaten family lives and societal values, strong motivators for change in preparation for future outbreaks and epidemics virtually certain to occur. The world goal is to prevent geospatially limited episodes from developing into the next global pandemic.

Mobile testing

Enhancing fundamental knowledge and practical expertise of those working in mobile testing modes, such as vans equipped with portable instruments, will help meet urgent demands for geospatially optimizing containment of COVID-19 and other highly infectious diseases.

Methods

Principles and strategies for pandemic response were derived from lessons learned following the COVID-19 outbreak in Wuhan, China in 2019 and its early spread to America and Southeast Asia (3-5), and before that, from the Ebola epidemic that devastated Western Africa in 2014 and still alarms the continent with recurrences in the Democratic Republic of the Congo and elsewhere (6-10).

Learning objectives

Essential learning objectives were extracted from an original lecture (~500 slides) and workshop course for POC operators and coordinators created for limited-resource settings by Kost et al. **(11)** This practicum has been taught worldwide for several years, typically in the format of two morning lectures followed by an afternoon hands-on workshop (wet lab) where students using POC devices generate real-time results.

Mathematical and geospatial optimization

Designs for public health POCT curricula were aided by quantitative mathematical and pattern recognition analyses of COVID-19 diagnostics and POC geospatial approaches applied to the current pandemic, which were published open access in the Archives of Pathology and Laboratory Medicine (12-14) and Frontiers of Public Health (15), respectively.

POCT IN PUBLIC HEALTH EDUCATION

A national survey of public health institutions by Kost et al. (1) identified future directions for POCT curricula in public health. The survey showed absence of instruction, hands-on training, and accredited courses in POCT in American

colleges, schools, and programs of public health education. Public health certification requirements and textbooks generally do not include POCT instruction.

In the national survey (1), the topic, "POC HIV/HCV ED" testing, appeared in only one course, and "POC diagnostics in local clinics," in one other. Only one book, Global Point of Care: Strategies for Disasters, Emergencies, and Public Health Resilience (16), and one online course on public health preparedness (17) address POCT for disaster and public health crisis intervention.

A 2021 review of PubMed searches and worldwide web searches revealed public health educational institutions have not yet incorporated POCT in curricula. Public health curricula do not address POCT in isolation units during quarantine or societal implementation of POCT broadly in communities, despite unequivocally proven need for POC strategies that enhance standards of care during the current COVID-19 pandemic (3).

PUBLIC HEALTH CURRICULA

Tables 1 through 4 present integrated modular sets of POCT learning objectives customized for public health students. Table 1 learning objectives focus on the mission and the basic principles of POCT (1). Sections II. A. and B. build technical skills. Collaboration with clinical chemists, laboratory scientists, clinical pathologists, inventors, and entrepreneurs will ensure high quality instruction in POC technologies and their design. A recent paper in this journal provided collaborative guidance for training and competency in POCT (18).

Sections II and III in Table 2 cover the general use of POCT in health maintenance, noncommunicable disease, and communicable threats (1). Section IV emphasizes how to position POCT in small-world networks (19) and the use of geographic information systems (20). The reader can refer to the papers and chapters cited for details that will aid instruction.

Table 3, titled Public Health Preparedness and Emergency Management, includes a special Section I.B. of learning objectives addressing POC strategies for COVID-19. It also references the Clinical and Laboratory Standards Institute guidance for Emergency Use Authorization (EUA) tests (21), because the FDA EUA process has been used extensively for COVID-19 diagnostics implemented in the United States. The learning objectives include hands-on workshops in Section I.C. Several of the entries could be assigned as timely Master of Public Health theses.

Table 4 enhances opportunities to embed POC principles and practice in standards, policy, guidelines, project management, and value propositions, so as to form infrastructure for sustainable funding and perpetual improvement in rapid response (1). Please also see Kost (14) for a summary of standards of care guidelines specifically for the use of COVID-19 rapid antigen tests, which have become available worldwide in the fight against COVID-19.

DISCUSSION AND CONCLUSIONS

Point-of-care testing is inherently fast, intrinsically spatial, and immediately actionable. It adds value by decreasing therapeutic turnaround time, speeding decision-making, and quickly enabling correct treatment. The mobility of POCT reduces risk by gatewaying testing before air travel, for facilitated immigration, at drive-ins/-ups/-throughs, in walk-bys and pharmacies, and for other optimal spatially isolated testing sites crucial to economic opening, safe spacing (social distancing), and successful tackling of risky COVID-19 exposure.

Public health policies and guidelines should sustain POC rapid response, as recommended

by the logic map of Figure 1. Support can come from the Centers for Disease Prevention and Control, Food and Drug Administration, General Accountability Office, National Institutes of Health, the United Nations, United States Agency for International Development, World Health Organization, and non-government organizations (NGOs). Public health graduates populate these entities, so their awareness of POC strategies is vital to assimilation of POC strategies and their future planning.

The long-term challenge is to train adequate numbers of public health officials who can deliver diagnostic testing in communities worldwide. It makes sense to "train the trainers," that is, develop new cadres of public health graduates who will enthusiastically reach out to the community and train those at the bedside or onsite in the field in the principles and practice of POCT. Tables 1-4 provide the educational tools to do that.

Educators should modify accreditation standards, so that POCT knowledge will be validated in public health certification exams and sustained long-term to recraft the profession for this type of point-of-need response. Experts from several disciplines can participate productively extending their didactic efforts, methods, and inventions (22-27) in support of public health educators and their curricula.

The COVID-19 pandemic showed us that community contagion inevitably leads to outbreaks in convalescent care homes and deaths among the elderly and highly vulnerable. Therefore, high performance POCT (12-14) must be deployed for screening, triage, and contact tracing of both patients and staff with results immediately available on a daily basis. This represents just one example. Public health leadership can customize course content to meet local community needs for diagnostics by selecting topics from Tables 1-4.

Multiple crises tend to occur simultaneously, and when they do, fixed resources become disabled by electrical shutdowns, physical isolation, and supply failures (16). Communities designate or construct alternate care facilities and plan near-patient critical care testing in support of critically ill and quarantined patients. They implement action plans for patient isolation units close to or just outside emergency rooms to avoid contagion inside and install adjacent isolators and isolation laboratories with POC instrumentation.

Training of community health providers must include the use of PPE and, importantly, practice in operating POC devices while wearing PPE. Mobile vans can be equipped with molecular diagnostics that detect highly infectious threats with high sensitivity and specificity, as well as rapid antigen, antibody, and multiplex SARS-CoV-2 + Influenza A/B tests for COVID-19 (5). This will help avoid crowding emergency rooms with potentially contagious patients, because testing will minimize both false negatives (high sensitivity) who spread disease, and false positives (high specificity) who may be committed to units housing infected patients and become infected themselves (12-14).

Government agencies should provide adequate resources and funding (see Figure 1) to sustain community resilience and improve medical and economic outcomes. Grass roots knowledge and skills in POCT, such as awareness of how to improve emergency diagnostics on ambulances (28,29), will help public health leadership create regional safety, while coordinating point-of-impact testing and important countermeasures, such as vaccination and drive-through testing sites (30-33). In fact, POC coordinators, a new and emerging subspeciality professional group in the POC field, can share responsibility for problem-solving strategies at points of need in America and abroad (34).

Table 1 Curriculum and learning objectives—mission, principles, and practice

Section & topics	Learning objectives
Part I.	**Getting started—the mission**
Goals, objectives, and overview of uses in public health	• Define POCT as *testing at or near the site of care* and appreciate that the definition does not depend on the instrument format or size • Understand the fundamental goals and objectives of POCT for rapid and effective evidence-based decision making at points of need
Needs	• Introduce situations where POCT has proven benefits for public health • Describe the need for POCT in outbreaks, epidemics, and the current pandemic, as well as in disasters and complex public health crises • Understand the importance of generating fast results, so that triage can be performed efficiently and immediately
Companion Tests	• Why do POC monitoring of temperature, oxygen saturation, respiratory rate, and other key primary variables (e.g., d-Dimer) provide layers of protection, defense, and assessment along spatial care paths
Part II.	**Fundamental principles and practice of POC testing**
A. Technical	
Needs assessment	• Develop competency in needs assessment for POC diagnostics in public health • Apply to healthcare settings limited-resource countries
Instrument formats, selection, and validation	• Recognize basic formats for disposable, handheld, portable, and transportable POC technologies that perform *in vitro* testing • Describe disposable POC tests, including smartphone modules, and their advantages, disadvantages, and marginal cost-effectiveness • Have the ability to select and validate the correct instruments
Non-invasive monitoring versus *in vitro* diagnostic testing	• Consider the operating principles of non-invasive devices, namely pulse oximetry for monitoring of oxygen saturation, and continuous hemoglobin monitoring • Compare *in vivo, ex vivo*, and *in vitro* approaches and advantages

Specimen processing	• Contrast whole-blood versus plasma analysis, also dry blots • Outline specimen processing and suitable sample types for testing in the field, primary care, and emergency room • Review special requirements associated with isolation laboratories
Quality assurance (QA), quality control (QC), and proficiency testing (PT)	• Identify "waived tests" under CLIA '88 and compare other POC tests • Know the definition and importance of quality assurance, including internal quality control and external quality assessment • Learn the five basic elements of the individualized quality control plan (IQCP), including environmental stress; how to customize QA, QC, and PT; and the importance of continuous quality improvement • Recognize confounding factors in diagnostic testing
Environmental stresses	• Overview the effects of environmental stresses on POC instruments and reagents, and how to avoid adverse consequences • Study methods for modulating environmental conditions for POC reagent storage and transportation
Multiplex molecular diagnostics	• Gain a basic appreciation of multiplex assays used for the detection of viruses, bacteria, and fungi, that is, pathogen detection • List advantages, disadvantages, costs, and limitations • Show examples of current POC disposable tests and instruments commercially available
B. Design & build	
Design criteria	• Read WHO and other POC device performance specifications
Commercialization	• Understand custom POC test clusters, basic manufacturing requirements, commercialization processes, and timelines
Regulatory oversight	• Review routine FDA 510(K) clearance and pre-market approval (PMA) • Outline the FDA system of classification of diagnostic tests (i.e., CLIA-waived, moderately complex, and complex) and the criteria for home testing. • Assess the ramifications for implementation, personnel, and use
FDA and WHO emergency use declarations	• Study the process, legal requirements, and terms of FDA emergency use authorizations (EUAs) and WHO emergency use assessment and listings (EUALs) • Locate EUA and EUAL listings and documentation of tests on the web

Accreditation options	• Understand the definition of accreditation and why an organization would engage in it • Discuss the main considerations and steps leading to accreditation • Consider inspections options for POCT [e.g., College of American Pathologists, Joint Commission, and CMS (for waived testing)]
Part III.	**Practicum**
Device hands-on experience	• Demonstrate CLIA '88 waived and moderately complex POC tests • Learn how to perform common POC tests, how to operate mobile POC instruments, and security features (e.g., UN & PW) • Watch demonstration videos of transportable whole-blood analyzers and test clusters for critical care and support of patients in isolation
Results interpretation	• Use case studies to demonstrate how to interpret basic test results
Performance evaluation	• Attend a workshop illustrating POC performance evaluation, such as regression analysis, Bland-Altman plots, and locally-smoothed (LS) median absolute difference ("LS-MAD") curves and maximum absolute difference ("LS-MaxAD") curves
Trouble shooting	• Gain experience at trouble shooting POC tests and devices • See examples of error codes and how to respond to them
Establishing a POC program	• Understand the steps necessary to establish a successful POC testing program

Table 2 Curriculum and learning objectives—public health sciences

Section & topics	Learning objectives
Part I.	**Integration of POC and public health expertise**
Roles of public health personnel and POC Coordinators	• Recognize the benefits of teamwork among public health practitioners, POC Coordinators, reference laboratories, and clinical laboratories • Develop personnel resources and a database of skill sets in advance of disasters, emergencies, complex crises, and epidemics • Understand that public health students and professionals could become POC Coordinators

Training, credentialing, and assuring competency	• List and analyze approaches to multidisciplinary credentialing • Specify requirements for maintaining competency and annual reviews • Learn how to document competency of Disaster Medical Assistance Teams (DMATs) and other first responders
Part II.	**Health maintenance and noncommunicable diseases (examples)**
Pregnancy	• Explore sensitivity, timing, and interferences, and the technical differences in disposable urine tests versus plasma assays
Prediabetes and diabetes	• Appreciate why plasma glucose standardization is necessary for consistent performance of blood glucose meters • Understand the role of POC HbA1c testing in the diagnosis and monitoring of prediabetes versus diabetes • Develop patient plans for self-testing of capillary whole-blood glucose • Correlate prevalence, demographics, and public health implications of evidence-based POC diagnosis in poor and rich nations
Acute coronary syndromes and acute myocardial infarction	• Study Spatial Care PathsTM (SCPs) for rapid home rescue of patients with acute chest pain • Apply evidence-based medicine (EBM) and learn why current POC cardiac troponin (cTn) tests are limited to ruling in (not ruling out) acute myocardial infarction • Read about prehospital diagnosis using POC cTn on ambulances • Strive to use POC cTn in rural areas to eliminate social inequity by rapidly diagnosing acute myocardial infarction and starting intervention
Part III.	**Communicable diseases (examples)**
HIV	• Appreciate the POC methods of screening for HIV, including pregnant women for prevention of transmission and algorithms for newborns • Study the advantages of simultaneous multiplex testing for concurrent diseases, such as TB
Influenza A and B	• Apply EBM principles to influenza testing and understand predictive values and their use from the viewpoint of the primary care physician

	• See examples of portable CLIA-waived instruments (Liat, Roche Diagnostics; Alere-i, Abbott) useful during flu season
Malaria	• Review new POC tests (e.g., fingerprick Ag Plasmodium falciparum) and uses in Africa and other endemic areas
Strep throat screening	• Review primary care practices related to screening • Understand necessary follow-up testing
Tuberculosis and resistance testing	• Cover instrumentation for TB diagnosis and resistance testing [e.g., the GeneXpert MTB/RIF test as a marker for multidrug resistant TB (MDR TB)], by drawing on the foregoing instruction in molecular diagnostics • Establish appropriate settings and conditions for testing • List and abate environmental stresses (e.g., temperature and dust)
Part IV.	**Geospatial science & geographic information systems (GISs)**
Small-world networks (SWNs)	• Define, illustrate, and analyze healthcare SWNs • Set the stage for community public health practice using POCT in optimized SWN healthcare delivery systems
GIS applications to health systems	• Explain how to set up and analyze a GIS • Establish SCPs within SWNs for rapid diagnosis and treatment • Assess the impact of GIS analysis of SWNs and SCPs • Integrate smartphone POCT-GIS for sentinel case tracking

Table 3 Curriculum and learning objectives—public health preparedness, emergency management, and the COVID-19 crisis

Section & topics	Learning objectives
Part I.	**Preparedness for outbreaks, epidemics, and isolation**
A. Test metrics	
Dynamic evidence-based medicine	• Compare sensitivity, specificity, and predictive values of POC tests • Point out that false negatives, FN(t), are a function of time, and therefore, sensitivity and the ability to rule out disease are dynamic characteristics when testing patients with evolving infections • Explain why 95% confidence intervals for diagnostics introduce peaks and valleys of uncertainty that vary with prevalence for infectious targets

B. Past perspective	
Ebola virus and other highly infectious diseases	• Document how the 2014-16 Ebola virus disease epidemic and cases entering the U.S. proved unequivocally the need for POCT • Overview how POCT could have curtailed the 2014-16 epidemic • Compare how rapid response limited the recent 2017+ outbreaks • Survey POC technologies available for Ebola virus disease and other high-risk pathogens
B. COVID-19 pandemic	**Special section**
Metrics of testing	• Compare and contrast molecular diagnostics, antigen assays, and antibody tests used to diagnose COVID-19, plot temporal trends, detect stealth infections, and judge immunity, including detection of variants (e.g., Delta) • Identify how sensitivity and specificity impact PPV, NPV, PV GM2, FP/TP, FN/TN, false omission rates, and other test metrics in settings of low, moderate, and high prevalence • Observe that patients with false negative results can spread SARS-CoV-2 unknowingly, and false positives can place people at danger when quarantined with who have COVID-19 • Show how uncertainty, in terms of 95% confidence intervals, impact test results
FDA Emergency Use Authorizations (EUAs)	• Outline FDA procedures and criteria for obtaining EUAs for COVID-19 tests, visit the FDA EUA website (https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas), and inspect authorization documents
Management of EUA tests	• Consult Clinical and Laboratory Standards Institute white paper EP43-Ed1, "Implementing a Laboratory Test Under Emergency Conditions," for method establishment, guidance on method implementation, and general management of EUA tests, including their potential retirement
Safe spacing & contagion	• Learn mobile POC strategies, such as drive-up/drive-through testing, kiosks at points of need, elderly access, home self-testing, and other safe approaches • Describe the role of POCT in contact tracing, its effectiveness or lack thereof, and the economics of pursuing superspreaders, reinfections, and herd immunity

Maximize the effectiveness of rapid antigen tests (RAgTs) and empower without intimidating	• Predictive value performance patterns suggest Tier 2 — positive percent agreement (sensitivity) of 95% and negative percent agreement specificity) of 97.5% — should become the minimum performance threshold for RAgTs. See reference 14 for details of tiers, performance patterns, and other details of RAgT clinical use. • Consider the current community prevalence and its impact on test results, especially in the high range of prevalence. • Self- and home test using a RAgT kit as soon as signs or symptoms arise and within the first 3 to 5 days for optimal detection of SARS-CoV-2. • When self- and home testing, repeat the test at 36 hours and follow the protocol specified by the manufacturer. Repeat testing will improve the performance of low and sub-tier tests. • Establish performance metrics (e.g., PPA, NPA, CI, and LOD) in diverse large multicenter populations with a full range of SARS-CoV-2 viral loads. Explicitly characterize the reference method (e.g., Ct brackets). • Harmonize preanalytical and assay methods. Do not compare to an inferior test. • Test free of charge everywhere, anytime. Test at home, when traveling, or any place in the world, and use the results to avoid and manage risk, not to punish. • When a person is not vaccinated, test weekly with a PCR assay or twice per week using a dual test RAgT kit if in the workplace, university, or similar environments. • Avoid stigmatizing positive test results by requiring unnecessary prolonged quarantine or shameful detention. Do not use test results to intimidate. Instead, optimize human resources. • End quarantine when test results turn negative. Allow work and other activities to resume with minimal personal and economic loss. • Create a positive and reassuring social milieu, a point of care culture of self-motivated frequent testing, so that empowered individuals can stop variant outbreaks and avoid spread to highly vulnerable people. • Expect COVID-19 to become endemic worldwide. Adapt by vaccinating, testing, and empowering. Diagnostic testing allows us to calibrate and manage our own personal risk.

C. Workshops	Highly infectious diseases
Personal protective equipment (PPE)	• Don PPE and practice performing POC tests, then doff the PPE and show that work was performed without personal exposure
Isolation laboratory and quarantine	• Be able to read floor plans, design an isolation laboratory, equip it with POCT, and route specimen workflow • Understand specifications for biosafety cabinets and limits to performing molecular diagnostics and POC tests within them • Identify special aspects of personnel training and protection
Spatial care pathsTM	• Demonstrate sentinel case discovery, 911 intent, and fastest rescue routes in healthcare SWNs • Place POCT to optimize efficiency and effectiveness
IQCP, its five key components, and plan design	• Practice designing individualized quality control plans (IQCP) • Remember the five components of the testing process: specimen, test system, reagent, environment, and testing personnel • Sketch out an IQCP for POCT in an isolation laboratory associated with a hospital and in an alternate care facility
Global pandemic preparedness	• Write a summary of POC strategies used in different countries to mitigate the COVID-19 pandemic and avoid saturation of hospital resources, such as ICU beds • Describe how POCT pivots community resources to optimize public health planning in the Unites States, then compare limited-resource settings • Define the specific roles of molecular diagnostics, antigen assays, and antibody tests, and which are available in portable or hand-held formats for testing onsite • Consider the economic tradeoffs of lockdowns, testing, and vaccination
Part II.	**Disasters, emergencies, complex crises, and rapid response**
Disaster caches and complex crises	• List the test clusters in DMAT POCT caches, the three US sites of storage, personnel training, and regional deployment, including Alaska and Hawaii • Recognize necessary steps in opening and using the compact and larger laboratory caches, test clusters, and their different purposes • Review the basics of specimen collection and sample preparation, including for infectious diseases, under challenging field conditions

	• Recognize the analytical limitations of POCT under disaster and complex crisis conditions
Performance standards	• Establish QC criteria necessary to complete before using POC devices from caches in the field during emergencies and disasters • Develop backup procedures in case of QA failures • Know National Disaster Medical System routines for maintaining high levels of performance when using POCT from caches in the field
Telehealth	• Gain familiarity with field connectivity and telecommunications
Alternate care facilities	• Integrate DMAT resources with community alternate care facilities
Bioterrorism	• Be aware of major threats, methods of detection, containment

Table 4 Curriculum and learning objectives—public health preparedness, emergency management, and the COVID-19 crisis

Section & topics	Learning objectives
Part I.	**Standards, policy, and guidelines**
A. Lectures	
International Organization for Standardization (ISO)	• Outline the purpose and contents of ISO 22870:2016, "Point-of-care testing: Requirements for quality and competence," and associated standards (e.g., ISO 15189:2012, Medical laboratories)
CDC, FDA, and WHO	• Review the guidelines and documents published by the CDC, FDA, and WHO for POC needs, technologies, and public health response
General Accountability Office (GAO)	• Analyze recent General GAO reports, webcasts, and documents regarding POC technologies for epidemics, molecular diagnostics, and cost-effective healthcare systems in the U.S. and abroad
Global status	• Compare and contrast national POCT policy and guidelines that have been established in Malaysia and Thailand, and their advantages and shortcomings (e.g., lack of disaster POC)
B. Workshops	
Procedures	• Understand the necessity for a set of written policies and procedures for POC testing • Be able to identify the core content of a policy or procedure

Policy and guidelines workshop	• Give learners the opportunity to draft an outline of the contents of national POCT policy and guidelines for a limited-resource country
Part II.	**Project management and POC value propositions**
Project management and the POC committee	• Understand the basic principles of project management • Consider how to analyze and effectively manage stakeholders • Understand the importance of the POC committee, anticipatory planning, and preparation for community projects
How to write a business case and develop value propositions	• Understand what information should be included in a business case • Be able to analyze the cost-effectiveness and value of POC testing • Identify key issues to address when implementing a new POC service
Part III.	**Global and future vision**
Course summary	• Recap what we have learned and what we can do with our knowledge to improve public preparedness, response, and health outcomes
Learner presentations	• Have teams of learners share studies of POC applications with which they have personal experience or have gleaned from literature
Future vision	• Understand the role of POC technologies in future public health initiatives, disaster preparedness, and stopping spread of outbreaks of highly infectious diseases in America and other countries • Place POCT on vans, ambulances, ships, aircraft, space flights, space stations, and planetary colonies • Realize that the principles and practice of POCT will evolve to maintain high standards of care adapted for mobile and remote settings as well as potential extraterrestrial life and encounters

The COVID-19 pandemic called us all to action, action that can be sustained through creative public health education in POCT. Knowledge learned, taught, and shared worldwide will help fill resilience gaps as the Delta variant becomes a global endemic disease and ubiquitous POCT to deal with it, the new normal.

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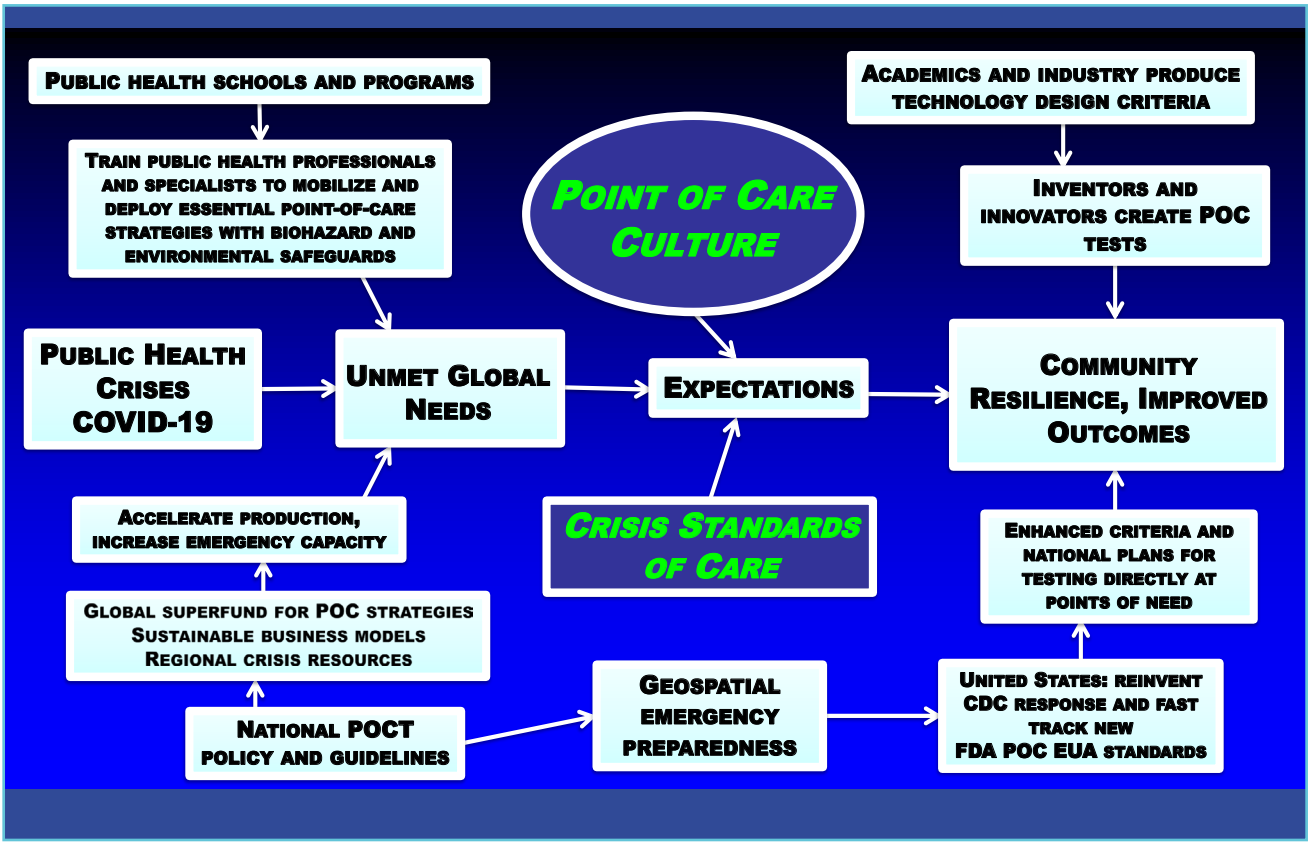
Figure 1 The incorporation of POC knowledge, skills, and culture in public health will lead to community resilience and improved outcomes — an integrative roadmap



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In this issue: Focusing on POCT

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Synergy between point-of-care testing and laboratory consolidations

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ABSTRACT

Central or area laboratories will offer an improved number of diagnostic testing services, where drivers for change will involve chronic disease clinical care for an increasingly older population, new emerging diagnostic technologies and personalized medicine. Higher automation quality and ever more diagnostic field integration will lead to higher productivity by means of an improved throughput. At the same time Point of Care Testing (POCT) site of patient care allows for timely medical assessment, which can lead to improved patient outcomes, more effectiveness and patient satisfaction. POCT test introduction in clinical practice should be assessed by an outcome-based policy to avoid adverse events, failure to diagnose providing appropriate timed treatment. The use of POCT devices does not only require technological considerations for the production and management of acceptable tests possibly managed by central laboratory, but also implicates a shift in diagnostic practice across all health organizations. The interaction between laboratory professionals and clinicians will

be enriched with new methods of evaluation of patient needs in the internet of things and mobile Health worlds, where boundaries between POCT and central laboratory or hospital and primary healthcare will no longer exist and where all data can be shared and disseminated among stakeholders in the healthcare system.

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CENTRALIZED, AREA, NETWORK OR HUB AND SPOKE LABORATORY SYSTEMS

Routine central laboratory has developed increasingly automated and effective instrumentation, including clinical chemistry, immunochemistry, common haematology testing and even very complex assays, executed using high throughput instruments following sophisticated automation. In the near future, comprehensive central or area laboratories of consolidated assay testing and larger laboratories will be most probable. The centralized clinical laboratory will offer an improved number of diagnostic testing services, where drivers for change will involve chronic disease clinical care for an increasingly older population, new emerging diagnostic technologies and personalized medicine. Higher automation quality and ever more diagnostic field integration will lead to higher productivity by means of an improved throughput. These goals are drivers for technological and management development of a centralized, area, network or hub and spoke laboratory systems, depending on the healthcare organization. Future health care and laboratory systems will have to deal with similar challenges as we face today, which may be summarized by the concept of "do more with less." The majority of tests will increasingly be performed in consolidated, high-throughput laboratories, because high analytical performance and cost efficiency cannot be fitted by current Point of Care Testing (POCT) technologies or other diagnostic systems. Current innovation in laboratory technologies is due to the availability of automation and development of analytical instruments, distributed across all disciplines of laboratory medicine. However, if Laboratory Medicine not only provides medical test results, but also helpful information and knowledge to clinicians and other stakeholders to assist decision making for individual patient optimal health outcome, the value of the laboratory is outside the laboratory. This is and will be of increasing value, as the assessments of the impact of medical testing on health outcomes will be a valuable proposition for laboratory medicine. This involves the correct and appropriate utilisation of delivered medical testing, where health results, operational and/or economic benefits are extended across the full clinical care pathway, focusing the interests of all stake holders (1). This aim is important in creating a leading role for laboratory medicine in the development of an effective and valuable healthcare system, as the most outstanding value in care is measured in terms of patient outcome. In this context, the laboratory achieves its best value when the patient successfully completes the diagnostic clinical care pathway. One of the values of laboratory medicine is based on how medical test results change the speed with which the patient completes the care pathway, by providing timely information, empowering clinicians or other stakeholders to make better and fast decisions about patients' care (2).

KEY DRIVER TO POCT IMPLEMENTATION

Over the past few decades, POCT has been one of the fastest growing disciplines in clinical laboratory medicine, equivalent and parallel to laboratory centralization. POCT is the execution of testing outside the clinical laboratory, near the patient or at the site of patient care. POCT devices are more widely used, both in acute and chronic patient management, inside the hospitals and in primary healthcare settings.

POCT where implemented both in critical care settings/emergency departments and in primary care settings, the assays are performed by nonlaboratory staff. Fast test results at the POCT site of patient care allows for timely medical assessment, which can lead to improved patient outcomes, more effective efficiencies and patient satisfaction (3). Since POCT activities are performed by non-laboratory staff who are unskilled in laboratory practices, one of the main challenges for POCT is the monitoring and management of quality assurance and regulatory compliance. The key driver to POCT implementation is the concept that clinical decision making may be delayed when samples are sent to the clinical laboratory when POCT is able to offer fast results closer to the patient, empowering medical decision making directly. The main endorsement for running POCT depends upon evidence which validates that a timelier result or shorter turnaround time is capable of influencing clinical improvement in decision making, when related with the central laboratory test delivery. In the last four decades, since POCT was adopted for the self-monitoring of blood glucose levels by diabetic subjects, various new POCT methodologies have become accessible, assisting the clinician in obtaining fast results to start treatment more rapidly. POCT seems to reduce pre analytical errors, where in laboratory medicine the higher number of errors happen in this phase (4,5). However, POCT is prone to errors in the analytical phase, due to the management of POCT instruments by staff unskilled in laboratory medicine. Conversely, the analytical phase has the smallest number of errors in laboratory medicine. In some settings, particularly remote rural environments and conditions, a central laboratory would be located at a great distance and the time to availability of some tests would not be acceptable. By contrast, in the Emergency Department, the availability of more rapid results with POCT is of value, despite the close location of the laboratory. POCT availability is just a means of better care delivery, as other barriers may be important to the implementation of care. Many reviews have applied principles of evidence-based laboratory medicine, seeking high quality systematic reviews and meta-analyses, to find the best possible evidence to support the question of whether POCT gives any advantage in clinical decision making in different scenarios (6) when compared to central laboratory.

THE MAIN THEORETICAL ADVANTAGE OF POCT TO SUPPORT CARE DELIVERY NEARER TO PATIENTS

In primary care there is an increasing focus on the need to encourage a more integrated healthcare attitude to support care delivery nearer to home, to improve not only patient satisfaction but health outcome in primary care. This trend has been matched by a requirement for innovative patient-centred care models (7), driven by the need to decrease rates of inappropriate or unplanned hospital admissions, better care for old patients with long-term chronic conditions and possibly cost containment. One means of realizing care closer to home is the implementation of POCT testing in course of a single routine appointment, supporting the hypothesis that this might decrease additional testing elsewhere, reiteration of visits or further medical appointments due to diagnostic uncertainty. POCT technology improvements have enabled most POCT devices with the knowhow to connect to the laboratory information system (LIS) and electronic medical records (EMR). Therefore POCT performance, when integrated in central medical laboratory activities, are becoming increasingly crucial as hospitals and healthcare systems are undertaking consolidation and harmonization by a continuous interaction (8) to promote the best utilization of diagnostic information and reporting. Some authors

recognize the POCT medical culture as one of the most important characteristics for reducing medical poverty and to design innovative and novel solutions at points of need, worldwide. In this light handheld, pocket-size and connected POCT tools and smartphone diagnostic devices will be used among populations, offering a new vision of healthcare, as an expected element of a highly informed everyday human lifestyle. Practicing point of care in the context of local medical culture is the final frontline and, if positively investigated, will become a 21st Century outstanding achievement (9). It is well recognized that the implementation of POCT is successful when the assay is by itself helpful for the medical decision-making process and does not need additional tests for confirmation from a central laboratory, otherwise the time benefit of POCT is minimal or ineffective. The main theoretical advantage of POCT is early and appropriate aid to diagnosis and treatment, but few studies are available about how POCT results influence clinical decision making. In the case of some immunoassays, POCT results seem to be reliable and accurate, such as for troponin, brain natriuretic peptide and C-reactive protein assays with satisfactory analytical performance together with an excellent feasibility, proposing them to be a consistent tool to be used in clinical practice. However, data and consequently derived evidence regarding clinical outcomes are lacking (10,11). There are many studies highlighting the agreement between POCT and central laboratory in terms of analytical and diagnostic accuracy, but results both on patient management and patient outcomes have not been consistently explored. Evaluation of patient outcomes is a key issue in the decision to implement POCT testing in place of central laboratory testing, and evidence to support this decision making is usually poor (12).

THE DEVELOPMENT OF A VALUE PROPOSITION FOR MEDICAL TESTING IN POCT OR A CENTRALIZED LABORATORY

Some critical steps including key points for discussion and evaluation, have been devised for the development of a value proposition for medical testing in POCT or a centralized laboratory as advised by the IFCC-Emerging Technologies Division (2).

1. What is the unmet clinical need resolved by POCT that cannot be resolved by central laboratory?

The unmet need of the medical test under investigation requires precise and clear definition of the clinical presentation, test impact and the setting of care, as for example the timely delivery of troponin to rule out suspected acute coronary syndrome or myocardial infarction, inside or outside the hospital setting. [13].

2. What is the clinical pathway in which the POCT is implemented?

The evaluation should focus on how test results improve clinical decision-making, patient management, medical appointments (urgent and non-urgent) and care process efficacy and efficiency. The appraisal should evaluate the clinical setting and report on how POCT medical test results are used, if the test is intended for diagnosis, for monitoring disease progression or for prognosis. Its position and role in the clinical pathway should be defined, such as whether it is a new test, an additional test, a replacement test or if it is used in patient triage. The clinical decision influenced by the medical test result and its impact on patient management needs to be clearly outlined, such as whether POCT testing is used to guide a therapeutic or other intervention.

3. What are the POCT test benefits vs central laboratory test?

The benefits of timed medical test assay to resolve the patient clinical need by robust and well demonstrated data, possibly based on clinical trials of POCT medical tests under evaluation, should be studied. The potential benefits derived from introducing POCT testing is of pivotal importance and should include the measurement of clinical, operational and economic outcomes, highlighting the reduction in time for patients to complete the clinical care pathway. Potential harms arising from the use of POCT medical test should be also identified.

4. Who are the POCT stakeholders?

The stakeholders, including the patient, the clinical team including laboratory medicine the healthcare purchaser and healthcare policymakers should be identified. Analyses of costs and benefits need to include the impact for each of these actors and stakeholders. Utilisation of health economic outcomes research is needed to guide the introduction of POCT new tests based on a firm foundation of evidence.

The principles of health technology assessment (HTA) are already applied in laboratory medicine and POCT evaluation so promoting efficacy, efficiency in POCT implementation when necessary, by attention to productivity through technology and process innovations (14). In the evolution of HTA appraisal, as in the case of POCT, patients need to be involved, particularly at the early stages (15) in terms of care value perception. Areas for improvement include aim, setting, and focus on the full health system effects (16). Further POCT test introduction in clinical practice may be assessed by an outcome-based policy on testing-related diagnostic errors (17) for a more active selection of useful biomarkers to avoid adverse events, failure to diagnose providing appropriate timed treatment. In this light the development of high-quality recommendations on POCT versus central laboratory testing may result in common framework to promote harmonisation and risk management in diagnostic pathway as reported in Table 1.

The Test Evaluation Working Group (WG-TE) of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) proposed an outcome-focused approach that can be used to evaluate any medical test, irrespective of the purpose and role of testing to identify clinical management decisions, linking biomarker testing to health outcomes (18). A patient-centred method that can be used in POCT test assessment is The Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach to assess both the certainty in evidence and to develop recommendations (19). Desirable and undesirable effects need to be judged in comparison to the old or traditional laboratory test due to a new POCT testing. The use or misuse of tests for a specific clinical presentation in different professional settings affects equity of access to clinical care (20), test the cost-effectiveness of interventions should include the evaluation of the impact outside the laboratory and the downstream consequence. The great challenge is to identify the overall health care cost and not only the plan cost of the test itself (21).

THE USE OF POCT DEVICES IMPLICATES A SHIFT IN DIAGNOSTIC PRACTICE ACROSS ALL HEALTH ORGANIZATION CLINICAL GOVERNANCE IN LABORATORY MEDICINE

The use of POCT devices does not only require technological considerations for the production and management of acceptable tests, but also implicates a shift in diagnostic practice across all health organizations. A new design for a chronic care model supports the integration of POCT in primary healthcare by an iterative scheme

Table 1 Relationships between clinical-laboratory harmonization in the total testing process and the outcome-based approach in the case of POCT

Outcome-based approach to testing-related diagnostic errors	POCT vs central laboratory harmonization in laboratory medicine
Source: Processes external to the laboratory	**Initial and/or final steps of the total diagnostic process (outside the laboratory) POCT vs Central Lab**
▸ An inappropriate test is ordered POCT vs Central Laboratory ▸ An appropriate test is not ordered POCT vs Central Laboratory ▸ An appropriate test result is misapplied both Central Laboratory and POCT	▸ Selection of references biomarkers POCT vs Central Laboratory ▸ Appropriateness in POCT test request ▸ Appropriate Test timed interpretation and decision to be acted ▸ References population data base
Source: Internal processes (within the laboratory)	**Internal processes (within the laboratory or managed laboratory POCT)**
▸ An appropriate test is ordered, but a delay occurs somewhere in the total testing process, POCT value ▸ The result of an appropriately ordered test is inaccurate due to an inacceptable POCT or Central Laboratory Management	▸ Evaluation of pre-analytical sources and pre-analytical quality both in POCT and Central Laboratory ▸ Harmonization of currently available assays and analytical control practice by POCT testing and Central Laboratory

process, to optimize a care model based on dynamic integration of POCT into the network of care delivery to optimize the benefit of the diagnostic test performed. This care model design is based on the integration of POCT through the connectivity with primary care providers according to clinically approved guidelines. Therefore, the POCT results are managed by the clinical central laboratory, not only as technology assurance but also in the diagnostic information process. Laboratory medicine specialists are likely to take a lead in organizing and managing multidisciplinary teams and to undertake this clinical diagnostic processes in terms of clinical governance (22). The brain-to-brain loop describes the process from the physician's decision to request a diagnostic test up to the action due to the reported result. The integration of all diagnostic laboratory test, POCT or central laboratory test performed, into the care pathways,

by digitalization of care will have an important impact on this process. Effective chronic disease management needs the involvement of multidisciplinary teams, stimulating and encouraging a continuum between primary, secondary and tertiary sectors. New clinical governance framework may be based on an integrated diagnostic framework, where POCT and central laboratory data are fully combined with all patient data to allow not only traditional policy and programme of quality assurance, risk management, technology assessment but also integrated for shared disease management.

THE DIGITALIZATION OF HEALTHCARE AND LABORATORY MEDICINE

The interaction between laboratory professionals and clinicians will be enhanced by digitalization, internet of things and mobile Health worlds, where borders between POCT and central laboratory will no longer exist. The availability of diagnostic Artificial Intelligence (AI) support utilizing POCT results coupled by laboratory data may be of value at the hospital admission promoting an accurate and fast diagnosis fostering the expected outcome or assuring the best possible care at home after discharge from hospital. Now and in the near future, new generation of electronic medical record systems digitally connecting information from POCT, Central or area Laboratory and patient homes, is an emerging healthcare model as proposed by the report from the IFCC-Emerging Technologies Division (2). The central laboratory robotization, POCT extension strategy, big data and algorithmic recording and reporting by artificial intelligence will drive the presence of another brain-to-brain loop or the so-called Lundberg cycle, defined as the "Artificial Intelligence Brain" (23).

Currently health care debate leads to a healthcare reorganization strategy where the hospitals are devoted to the emergencies and intensive care management while chronic patient care is decentralized in community hospitals or near patient health structures or patient home managed by GP and/or nurses. In this light the laboratory diagnostic test may be a driver for best medical decision based on the interaction with all patient data derived by clinical history when available. This may be of value in home to hospital care as in the case of patient with acute disease, or in hospital to home in the case of low-level intensive care as in patient with chronic disease. The future balance between testing in central laboratories and testing at the point of care is difficult to predict accurately (24) as POCT or near-patient testing is now starting to look to laboratory testing with new mobile devices and online services in a new context of home bedside care or self-care.

The rise of AI and machine learning can allow the combination of data from different hospital settings, POCT, central laboratories and healthcare sites to promote the "learning" of predictive models (25,26) as distributed learning.

The availability of large population medical datasets opens the way to approaches based on the analysis of data to generate diagnostic hypotheses that can be confirmed by further test inside laboratory or outside. POCT area datasets merged by population diagnostic records derived by area or network laboratory data are means to develop real-word AI diagnostic support tools to improve the management of chronicity in primary near patient care. In this light the use of POCT technologies offer an opportunity to promote the best use of laboratory results even in absence of skilled physicians. This approach may be operated in remote, primary and secondary diagnostics.

In **remote diagnostic** to obtain information on symptoms and signs that can allow establishing the degree of urgency and the requirements for diagnostic tests, like POCT, based on data

already known possibly in primary care. This will also increase the positive predictive value for the POCT tests by pre-screening for patients that are more likely to have the condition assessed by POCT.

In primary diagnostics as reliable and cost competitive primary diagnostics support tool for common diseases by simplifying the diagnostic process. The AI may be helpful to advise still rare or uncommon diseases streamlining the process of obtaining the right diagnosis reducing the delays in the diagnosis.

In secondary and tertiary diagnostics by the application of AI integrated with secondary diagnostics tools, such as ECG, imaging, and all available population clinical data set. AI or diagnostic support tool will help to identify patients, who either do not get a diagnosis or in situation where the diagnosis is particularly difficult. This approach enables clinicians to make a diagnosis with increased accuracy significantly improving patient journey identifying complex cases where a precise diagnosis is difficult.

Synergies on Laboratory Medicine Department basis between POCT results with all real patient diagnostic data available as present in area Laboratory Information System repository will unlock AI based potential diagnostics support tools, providing quicker and more accurate and less expensive diagnosis.

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Cardiac biomarkers in COVID-19: a narrative review

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ABSTRACT

The diagnosis and risk stratification of coronavirus disease 2019 (COVID-19) is primarily based on discretionary use of laboratory resources. Several lines of evidence now attest that cardiovascular disease not only is a frequent complication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, but its pre-existence may increase the risk of morbidity, disability, and death in patients with COVID-19. To this end, routine assessment of biomarkers of cardiac injury (i.e., cardiac troponin I or T) and dysfunction (e.g., natriuretic peptides) has emerged as an almost essential practice in patients with moderate, severe, and critical COVID-19 illness. Therefore, this narrative review aims to provide an overview of cardiac involvement in patients with

SARS-CoV-2 infection as well as the clinical background for including cardiac biomarkers within specific panels of laboratory tests for managing COVID-19 patients.

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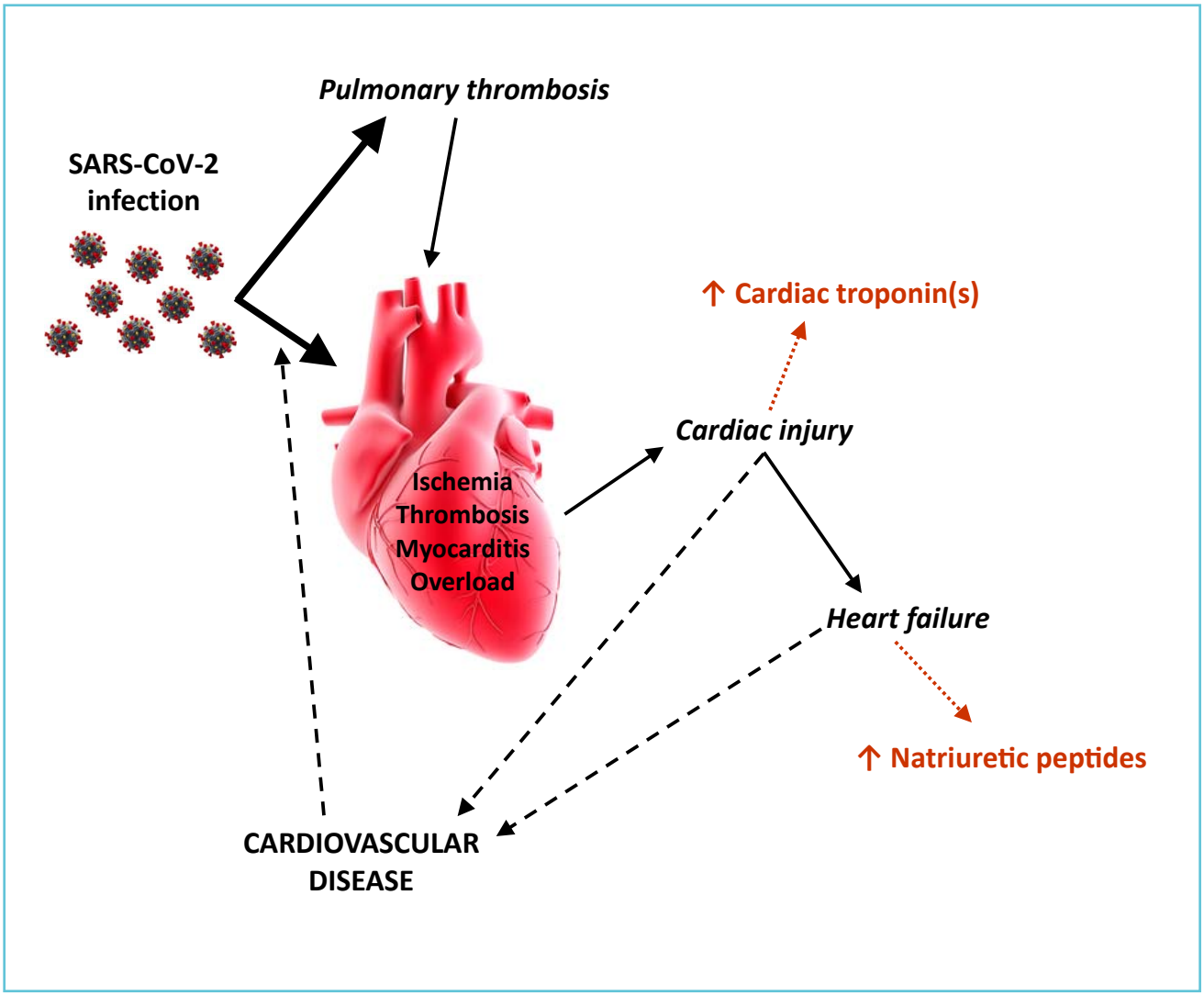
INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disorder caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which first appeared at the end of 2019 in Wuhan (China) and has since then assumed a pandemic proportion, causing several million deaths around the world so far [1]. One essential aspect in the pathogenesis of COVID-19 is that SARS-CoV-2 infection seems to generate the worst damage in people with one or more co-morbidities. Along with older age and male sex, cumulative Italian data suggests that the death rate in people without a single co-morbidity is around 2.8%, but then increases exponentially in those with 1, 2, and up to 3 co-morbidities [2]. Notably, cardiac pathologies, thus encompassing ischemic heart disease (IHD), atrial fibrillation, heart failure (HF), and hypertension, are the most important predictors of unfavorable COVID-19 outcome [2]. Data published by the US Centers for Disease Control and Prevention (CDC) are virtually overlapping. Specifically, an analysis of patients who died of COVID-19 in 2020 in the US has revealed a considerably high prevalence of cardiovascular disease (CVD), nearly 61%, not differing substantially across different age groups [3]. Interestingly, another comprehensive report published by the CDC highlighted that the burden of CVD was as high as 22% in people younger than 21 years who died from COVID-19 in the US [4]. To specifically address the impact of CVD on the prognosis of COVID-19 patients, a recent meta-analysis found that this preexisting condition was associated with a 3-fold higher risk of developing severe COVID-19 disease, as well as with 11-fold and 1.7-fold enhanced risk of mortality in all COVID-19 patients and those with severe disease, respectively [5].

Irrespective of the dramatic impact that pre-existing CVD may have on the clinical progression of SARS-CoV-2 infection, it is undeniable that COVID-19 itself may have a substantial impact on cardiac integrity and function, thus paving the way to the establishment of a potentially devastating vicious circle (Figure 1). It has now become rather clear that COVID-19 is not a single-organ, clear-cut pulmonary disorder, but is instead a gradually evolving pathology, characterized by a series of stages sustained by different biological mechanisms, as reviewed comprehensively elsewhere [6]. Briefly, while remaining mostly asymptomatic, or only mildly symptomatic, in the vast majority of subjects, in a variable percentage between 15-30% of subjects with SARS-CoV-2 infection the illness progresses towards a respiratory phase, whose hallmark is the development of an (often bilateral) interstitial pneumonia. An abnormal, almost exaggerated, immune and inflammatory response in some COVID-19 patients then paves the way to progression towards a subsequent phase, characterized by development of lung and systemic hyper-inflammation and gradual evolution towards lung and multiple organ injury, thus including heart and blood vessels [7]. In a low but still clinically meaningful number of patients (i.e., between 2-5%), the disease progresses into a further - highly critical - phase, where hyper-inflammation triggers activation of both primary and secondary hemostasis, which then leads to generation of intravascular coagulopathies manifesting as in situ pulmonary thrombosis, venous thromboembolism with deep vein thrombosis and/or pulmonary embolism, secondary thrombotic microangiopathy, or even disseminated intravascular coagulation [8].

Figure 1 Pathways of cardiac involvement in patients with severe acute respiratory infection coronavirus disease 2019 (COVID-19)



COVID-19 AND THE HEART

The risk of cardiac tissue injury in SARS-CoV-2 infection is certainly not surprising if one considers that similar myocardial damage can also be observed in patients with many other viral infections. For example, elevations of cardiac troponins have been found in up to 33% of patients with influenza, especially in those infected by more virulent strains such as H1N1, which belongs to the same family which caused the dramatic Spanish flu outbreak nearly 100 years ago [9]. The mechanisms of influenza-related cardiac injury are likely similar to those causing myocardial injury in patients with SARS-CoV-2 infection and involve either direct or indirect cardiac injury. It is also interesting to compare the incidence of cardiac injury in COVID-19. For example, a recent study found that a certain degree of cardiac involvement can be present in 21% of patients hospitalized for COVID-19, increasing to 50% in those with critical illness [10]. Cardiac injury was found to be more frequent in patients with COVID-19,

though its burden was not so dissimilar from that found in those with H1N1 infection (i.e., 50% vs. 39%). A variable cardiac involvement seems hence relatively frequent in patients with COVID-19. Another recent meta-analysis has estimated an incidence as high as 42% in patients with severe illness and 26% in those with milder disease [11]. It is then noteworthy that the development of cardiac injury in patients with SARS-CoV-2 infection was found to be associated with an over 10-fold higher risk of death [11].

Although the pathophysiology of cardiac injury in patients with COVID-19 is virtually multifactorial, patients may develop ischemic heart disease (IHD), which can cause either worsening of pre-existent IHD or alternatively present as an ex-novo consequence of the paradigmatic thromboinflammatory condition which characterizes SARS-CoV-2 infection. This was demonstrated in a study of patients with acute myocardial infarction (AMI) with or without COVID-19, showing that the burden of coronary atherosclerosis was lower in those with SARS-CoV-2 infection. At the same time, the presence of neutrophil extracellular traps (NETs) was markedly enhanced in coronary thrombi of such patients, thus suggesting a de-novo pathology [12]. It is then noteworthy that COVID-19 patients who develop ischemic cardiac injury have a much worse outcome than those without SARS-CoV-2 infection. A prospective international registry of acute coronary syndromes, which compared the outcome of AMI in patients with or without COVID-19, revealed that the mortality was over 3-fold higher in patients with SARS-CoV-2 infection, and was also associated with significantly longer hospitalization [13].

Along with the thrombotic/ischemic injury, it cannot be discounted that some patients may also develop a localized SARS-CoV-2 myocardial infection. A comprehensive assessment of COVID-19 associated myocarditis has been recently carried out by Bailey et al. [14], who found that SARS-CoV-2 can infect cardiomyocytes through an angiotensin-converting enzyme 2 (ACE2) and endosomal, cysteine protease-dependent pathway, which is then followed by enhanced cytokine production, sarcomere disassembly, and irreversible injury, up to cell death. Importantly, infection of cardiomyocytes by SARS-CoV-2 seems to reduce contractility due to sarcomere breakdown and cardiomyocyte necrosis.

Post-mortem studies have convincingly confirmed the risk of developing severe involvement of cardiac tissue in patients with severe/critical COVID-19 illness. A cases series of post-mortem analyses of patients who died from COVID-19 revealed that heart microthrombosis was present in as many as 80% of cases, and was virtually commonplace in those who died after severe/critical illness [15]. The rate of fibrin microthrombi in the heart was also nearly 3-fold higher than that observed in patients who died from influenza. At the same time, the prevalence of myocarditis was relatively low in both circumstances, though remaining nearly twice as high in COVID-19 patients. This evidence has then been confirmed in another study involving 40 patients who died from COVID-19 [16]. Despite a relatively low prevalence of baseline coronary artery disease, present in less than one-fourth of all patients, myocardial infection could be detected in 7% of all subjects, while focal myocyte necrosis was widespread, found in 80% of patients with COVID-19-associated myocardial injury. Importantly, another study published by Marfella and colleagues evidenced that nearly 85% of all patients who died with SARS-CoV-2 infection had thrombus specimens in cardiac arteries that were positive for viral RNA, thus confirming the important role played by SARS-CoV-2 in triggering ischemic and non-ischemic cardiac injury [17].

In summary, the origin of myocardial injury seems multifactorial in patients with severe COVID-19, involving myocarditis directly caused by SARS-CoV-2 infection, myocardial damage caused by thrombo-inflammation, Takotsubo syndrome, cardiac overload due to pulmonary thrombosis, along with type 1 and 2 AMI, the former due to obstruction of blood flow within coronary arteries, the latter due to an imbalance between oxygen demand and supply, which is in turn due to pneumonia-causing lower blood oxygenation, combined with fever and/or tachycardia driving increased oxygen demand (Figure 1) [18].

One of the most obvious consequences of the onset of myocardial injury in COVID-19 patients, besides the enhanced risk of mortality, is the risk of developing HF in the medium- and long-term period. A recent meta-analysis showed that the prevalence of this condition was around 20% in patients who recovered from COVID-19, and its presence was associated with an over 9-fold higher risk of death [19]. Irrespective of the cause, the gradual impairment of cardiac function appears an important risk factor for unfavorable outcomes in COVID-19 patients, with both impaired left ventricular ejection fraction and right ventricular dysfunction found to be important predictors of mechanical ventilation and/or all-cause mortality [20].

CARDIAC BIOMARKERS IN COVID-19

Cardiac troponins

Before exploring the importance of measuring cardiac biomarkers in COVID-19, a brief introduction may be necessary. Cardiac troponins are no longer considered the sole and unique biomarkers of myocardial injury, with their assessment extending far beyond diagnosing myocardial damage, now encompassing risk stratification of medium and long-term adverse outcomes. As largely proven, the concentration of cardiac troponins may increase as a consequence of a kaleidoscope of biological pathophysiologic pathways affecting the cardiac tissue such as ischemic, traumatic, toxic, and, last but not least, infectious insults, as well as being associated with several secondary cardiac damages as a consequence of renal failure, sepsis, cancer, pulmonary embolism, rhabdomyolysis, traumas, and burns, among others [21]. All the above mechanisms will variably lead to increased serum or plasma cardiac troponin concentration, depending on type and severity of the primary or secondary cardiac injury. Patients with increased values of cardiac troponins have a magnified risk of long-term morbidity and mortality in the general population, as well as in patients with specific pathologies, as demonstrated by a vast array of published meta-analyses, that we have recently reviewed elsewhere [22].

Hence, it is not surprising that a strong association has also been found between increased values of cardiac biomarkers, especially cardiac troponins, and unfavourable outcome of COVID-19. In fact, we demonstrated this very early in the COVID-19 pandemic. A recent meta-analysis showed that the values of cardiac troponin I were significantly higher in patients with severe COVID-19 illness, as well as in those who died. Cumulatively, an increased value of cardiac troponin I was found to be associated with an over 5-fold higher risk of developing severe illness [23]. Another updated meta-analysis has more recently confirmed that increased values of cardiac troponin I were associated with a remarkable 25-fold higher risk of death in patients with COVID-19 [24]. This evidence has been reported in many other studies, like that published by Kinght et al. [25], who found that up to 71% of all COVID-19 patients present with abnormal levels of cardiac troponins, and the in-hospital mortality rate of those with pathological values is as high as 41%. In those with

apparently unknown causes of cardiac injury investigated with cardiac magnetic resonance imaging (MRI), the injury was non-ischemic in nearly 38% of cases, ischemic in 17% of cases, and both ischemic and non-ischemic in 14% of cases. Notably, in those with known causes of cardiac injury, acute coronary syndrome, and pulmonary embolism were identified in 27% and 54% of cases.

Another important aspect has been highlighted in the study of Tanboğa and colleagues [26], showing that not only are cardiac troponin levels significant predictors of worse outcomes in patients with SARS-CoV-2 infection, but also that the relative increase in the concentration of this biomarker was directly related to negative disease progression and mortality, with such risk increasing from 1.2 folds for values marginally exceeding the upper reference limit, up to over 2.4 folds when cardiac troponin levels were 50-fold increased over the upper limit of normal. In the study conducted by Cunningham et al. [27], the prognostic impact of cardiac troponin has been investigated in more than 12,000 patients hospitalized for COVID-19. Besides the fact that the risk of death increased steadily across classes of cardiac troponin values, it was also interestingly found that such risk increased nearly exponentially in parallel with aging within each type of cardiac troponin (I or T) values. This implies that cardiac troponin values in COVID-19 patients, as in most other pathologies, should be interpreted considering the patient's age. Another interesting perspective to approach the role of cardiac troponins in COVID-19 encompasses the stratification of COVID-19 patients according to the severity of myocardial injury, followed by analysis of the relationship between cardiac troponin and outcome.

In a study by Salbach et al. [28] this approach revealed that cardiac troponin values gradually increased across different stages of severity of myocardial injury, and the primary endpoints raised in parallel across classes of injury severity, cardiac troponin, and D-dimer values. It is also noteworthy that an increased cardiac troponin value is indeed associated with worse cumulative outcome, but also correlates with a variety of adverse secondary endpoints. The study published by Shah et al. clearly shows that not only does the risk of in hospital-mortality increase across the four quartiles of cardiac troponin I, but a similar trend can be seen in the need for dialysis, mechanical ventilation and intensive care [29].

The extent of cardiac troponins elevation seems to be a significant prognostic factor in patients with COVID-19, but also its kinetics during hospital stay appears to have an important influence on the outcome. As shown in the study of Nuzzi et al. [30], COVID-19 patients with normal cardiac troponin values at admission, but who then displayed increasing concentrations, were found to have a higher risk of in-hospital mortality than those admitted with baseline elevation and stable levels afterward.

The substantial role of cardiac troponins in the comprehensive management of COVID-19 patients has also been highlighted by studies showing that incorporation of their values within simple algorithms may be efficiently used for predicting a vast array of unfavorable outcomes [31,32].

Such an important role played by cardiac troponin in COVID-19 has hence persuaded us to elaborate an algorithm that would make sense of their testing in patients with SARS-CoV-2 infection, for allowing the identification of those at higher risk of developing cardiac injury, either directly triggered by SARS-CoV-2, or as an indirect consequence of thromboinflammation, ischemia and/or myocarditis, as available elsewhere [33]. According to this model, nonevolving cardiac troponin values, along with normal levels of other biomarkers of inflammation or

cardiac dysfunction, may safely limit the necessity to perform other diagnostic investigations such as transthoracic echocardiography or stress testing in patients with suggestive signs or symptoms of cardiac involvement.

Natriuretic peptides (NPs)

Besides cardiac troponins, the clinical usefulness of measuring NPs in COVID-19, mostly encompassing the assessment of B-type natriuretic peptide (BNP) and NT-pro-BNP, has been broadly supported by clinical evidence. Patients with HF often have elevations in NPs, and those with pre-COVID-19 HF, both with reduced and preserved ejection fraction (EF) have marked increases in mortality with COVID-19, whereas just EF does not correlate with outcomes. In a meta-analysis by Zinellu et al. [34], significantly increased values of NPs were found in COVID-19 patients with different degrees of clinical severity compared to those with milder illness, without evidence of substantial differences between measuring BNP or NT-proBNP. Interestingly, unlike the evidence reported by Zinellu et al [34], De Falco and colleagues concluded that the diagnostic performance for predicting unfavorable COVID-19 outcomes, specifically, mortality risk, appears to be higher using NT-proBNP (area under the curve (AUC), 0.943) than BNP (AUC, 0.736), with a prognostic accuracy that was similar to that found for cardiac troponin I (AUC, 0.939) [35]. In an ensuing study by [27], the authors explored the prognostic role of NPs in predicting the outcome of over 12,000 patients hospitalized for COVID-19. Two exciting findings emerged from this work. First, the risk of death was directly dependent upon admission values of NPs, and, even more importantly, such risk increased nearly exponentially in parallel with aging.

It is then noteworthy that the assessment of cardiac biomarkers would not only help predict clinical outcomes of COVID-19 in the adult population, since Güllü et al. found that NPs and cardiac troponins are efficient predictors of development of the multisystem inflammatory syndrome in children, perhaps the most severe manifestation of COVID-19 in childhood [36].

Additional cardiac biomarkers

Although it is now undeniable that laboratory assessment of cardiac injury and function shall be almost entirely limited to assessment of cardiac troponins (either I or T) [37] and natriuretic peptides (either BNP or NT-proBNP) [38], evidence has been provided that some additional biomarkers may have a supportive role in stratifying the risk of COVID-19 patients. For example, Growth Differentiation Factor-15 (GDF-15) is another cardiovascular biomarker that may provide useful prognostic information in COVID-19. In a preliminary study published by Myhre et al. [39], the concentration of this biomarker was significantly correlated with the viral load and also positively associated with higher risk of unfavourable disease progression. It is noteworthy that its predictive value (AUC, 0.78) was found to be even higher than that of D-dimer (AUC, 0.63), cardiac troponin T (AUC, 0.63), NT-proBNP (AUC, 0.61), either expressed as baseline level or variation from the admission value. In a recent meta-analysis, we found that mid regional proadrenomedullin (MR-proADM), whose increased levels are commonplace in patients with critical illness, were significantly enhanced by 74% in COVID-19 patients with severe or critical disease compared to those with milder illness [40]. This evidence was confirmed in an ensuing study, which also showed that assessment of MR-proADM has an accuracy as high as 95% for predicting mortality in patients with COVID-19 [41]. Emerging evidence suggests that the assessment of soluble suppressor of tumorigenicity 2 (sST2) may also provide valuable insight into the progression of COVID-19, though large prospective clinical

studies to confirm its usefulness are still needed [42]. Finally, among the various red blood cells (RBC) parameters that may be useful for monitoring COVID-19 or predicting the risk of developing serious illness, the RBC distribution width (RDW) deserves special attention. In an original investigation, we assessed the prognostic performance of RDW in 49 COVID-19 patients, 16 with severe illness, 12 with severe acute kidney injury, and 8 needing renal replacement therapy [43]. In this population, elevated RDW significantly predicted all the unfavorable clinical endpoints and, specifically, was found to be associated with an over 9- and 16-fold higher risk of developing severe illness and acute kidney injury.

CONCLUSIONS

Several lines of evidence now support the inclusion of cardiac troponins and NPs as essential parameters within specific panels of laboratory tests for managing COVID-19 patients. These tests would enable a more timely diagnosis of cardiac injury, either direct or indirect, overall disease progress, as well as may help predict the risk of developing post-COVID-19 cardiac dysfunction.

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Circulating microRNA-126 as an independent risk predictor of coronary artery disease: a case-control study

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circulating biomarker, coronary artery disease, chest pain, microRNA

ABSTRACT

Context

Circulating microRNAs (miR) have revolutionized the field of molecular biology owing to their potential as a diagnostic as well as a prognostic biomarker of cardiovascular disease and dysfunctions. The present study aims to identify the circulating miR-126 and -122 as an independent risk predictors of coronary artery disease cases.

Methods and material

Blood samples were collected from coronary artery disease cases (n=100) and non-CAD cases (n=100). Serum RNA was isolated by Trizol method. MiR levels were measured by quantitative real-time polymerase chain reaction with the specific primer probe set.

Results

MiR-126 levels were significantly down-regulated in CAD cases compared to non-CAD cases (controls) (80.0% vs. 39.0%, χ^2=14.95, p<0.001). The level of miR-122 was significantly up-regulated in CAD cases in comparison to its non-CAD variant (14.0% vs. 63.0%, χ^2=21.23, p<0.001). Multivariate analysis found chest pain (OR=37.07, 95% CI=3.21-169.04, p=0.017) and miR-126 (OR=0.01, 95% CI=0.00-0.63, p=0.030) as independent risk predictors of CAD.

Conclusion

The results of our study show the potential of circulating miR-126 as a novel non-invasive biomarker in the risk prediction of CAD. Further unraveling of the role of miR-122 and miR-126 in the pathogenesis and progression of CAD will add to our understanding of the disease process leading to a new diagnostic approach.

Highlights

- Mir-122 and -126 significantly differentiate non CAD cases from angiographically proven CAD cases
- Chest pain and miR-126 might work as an independent risk predictor of coronary artery disease

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INTRODUCTION

Globally, cardiac disease is the predominant cause of death in the past several years. Multitude factors are associated with the disease; the major risk factors are hypertension, high cholesterol levels, unhealthy diet, obesity, diabetes, and addiction in different forms like alcohol and tobacco chewing.[1] Atherosclerosis and thrombosis due to unhealthy diet, lack of physical exercise, and hypertension ultimately lead to the development of coronary artery disease (CAD).[2] Diagnosis of CAD is based on the invasive coronary angiogram (CAG) and radiologic techniques such as an echocardiogram (Echo), computed tomography (CT), electrocardiogram (ECG), and exercise treadmill test (ETT). Several blood-based markers have been studied in the diagnosis of CAD but without any significant clinical result.[3] Circulating microRNAs (miRNAs) have revolutionized the field of molecular biology owing to their potential as diagnostic as well as prognostic biomarkers of cardiovascular diseases and dysfunctions. MicroRNAs are short, single-stranded non-coding RNAs that regulate cellular functions by degradation and translational repression of mRNAs. They assist in cell proliferation, differentiation, metabolism, apoptosis, development, aging, and in the pathophysiology of many diseases namely, oncogenesis, cardiovascular, and neurological disorders. Several studies have reported dysregulation of miRNAs in heart diseases such as myocardial infarction (MI), cardiac hypertrophy, fibrosis, and developmental heart disease. [4-7] Studies reported the change in the number of miRNAs in such pathological processes and the abnormal expressions of miRNAs are associated with a different type of heart disease. [8-10]

In earlier studies reduced plasma concentration of miR-126 in patients with heart failure (HF) compared to healthy controls were found to have an inverse co-relationship with brain natriuretic peptide (BNP), thus proving as a classic marker of HF in past studies. Higher levels of miR-126 indicated the better clinical condition of patients. Xiao Sun et al in 2012 demonstrated the relationship between miR-126 and LDL cholesterol in patients with or without CAD, which may have significant implications for identifying the potential role of miR-126 in cholesterol metabolism. [11] Different miRNAs related to cardiac origin have been studied to investigate the diagnostic potential. Reduced expression of miR-122 has been found in patients with MI. Thus, the current study was designed to assess

the levels of miR-126 and 122 in CAD cases for evaluating the risk predictors in coronary artery disease cases in India.

SUBJECTS AND METHODS

Study population

The study subjects were enrolled from January 2019 to December 2019. Coronary angiograms were evaluated by a clinician, who made a visual estimation of luminal narrowing in multiple segments based on the AHA/ACC classification of the coronary tree.[12] All the subjects fulfilling the inclusion criteria were enrolled for the study. All the cases were newly diagnosed. The blood sample was collected before the start of medication-related to the CAD and before the coronary stent implantation. Written informed consent was taken from the subjects. Approval to conduct the research was obtained from the Institutional Ethical Committee (approval number IEC95) before the start of the study and complies with the ethical principles for medical research involving human subjects, by the Declaration of Helsinki. Patients attending the OPD and who were not suffering from either detectable coronary stenosis or atherosclerotic vascular disease were considered as controls (CAD-ve). Patients were interviewed to collect information about their medical history and lifestyle habits. Risk factors were determined by a physician. Subjects were excluded from the study if they are affected by the hepatic failure, renal failure, abnormal liver function, hepatitis, cardiomyopathy, congenital heart disease, bleeding disorders, previous thoracic irradiation therapy, and malignant diseases.

Samples collection and serum isolation

Peripheral blood (3.5 ml) was collected from 200 cases in plain and EDTA vials (NOVAC, POLYMED, POLY MEDICURE LTD, India). Serum was separated by centrifugation of peripheral blood in plain vial at 1900g for 10 min, followed by a 10 min high-speed centrifugation at 16,000g and stored at -80°C until further processing.

Biochemical examination

Biochemical parameters including HbA1c (%) (D-10 Bio-Rad, USA), total cholesterol (TC), (mg/dl), triglyceride (TG), (mg/dl), high density lipoproteins (HDL-C) (mg/dl), low density lipoproteins (LDL-C) (mg/dl), very low density lipoproteins (VLDL-C) (mg/dl), folate II (nmol/L), Vitamin B12 (pg/ml), small dense low density lipoproteins (sdLDL) (mmol/l), and total homocysteine (HCY2) (umol/L) were recorded. All biochemical parameters were measured with a fully automated biochemical analyzer (ARCHITECT i2000SR, Abbott Diagnostic & Selectra ProXL, ELITech Group).

RNA isolation and cDNA synthesis

Total RNA was extracted using a Trizol-based miRNA isolation protocol (Invitrogen, Carlsbad, CA, USA) by the addition of 750µl of Trizol reagent to 250µl of plasma. The RNA concentrations were measured with a Nanodrop and cDNA was prepared using a commercially available MuLV reverse transcriptase kit (Cat. no. K1622, Thermo Fisher Scientific, USA). [13]

Quantitative real-time PCR

cDNA was amplified with specific primer sets miR-122 (hsa-miR-122-5p, Cat. no. 4427975), miR126 (hsa-miR-126-5p, Cat no. 4427975), and RNU6 (Cat no. 4427975). Quantitative real-time PCR (qRT PCR) was carried out using 7500 real-time PCR system (Applied Biosystems, USA) using TaqMan® Universal Master Mix II No UNG (Applied Biosystem, USA) according to the manufacturers' instructions. Data were normalized for RNU6 (housekeeping gene) expression by the comparative threshold cycle method. Duplicate Ct values were averaged, the relative expression levels of miR were calculated using

formula ΔCt = Ct[Target]-Ct[Housekeeping] and ΔΔCt = (ΔExp.)- (ΔControl) and got the -ΔΔCt log-fold-change and fold-changes were calculated for each miRNA. [13]

VEGF ELISA

Serum VEGF level (pg/ml) was determined by ELISA using RayBio Human VEGF ELISA kit and the reading were recorded by iMark™ microplate absorbance reader (BIOS) at 450 nm. The level of VEGF concentration (pg/ml) in cases was determined by comparing the OD of the samples with the standard curve.

Statistical analysis

Discrete (categorical) data are presented in number (n) and percentage (%). Categorical groups were compared by the chi-square (χ^2) test. Independent predictor(s) of CAD were assessed using univariate (crude or unadjusted odds ratio) and multivariate (adjusted odds ratio) binary logistic regression (BLR) analysis. Receiver operating characteristics (ROC) curve analysis was done to assess diagnostic accuracy (sensitivity and specificity) of markers (miR-122 and miR-126) for CAD assessment. Based on the data, a cut-off point was chosen, where the higher sensitivity and specificity were obtained (Data not shown). All continuous data were categorized into two groups (non-CAD and CAD cases). A two-tailed (α=2) $p<0.05$ was considered statistically significant. Analyses were performed on SPSS software (windows version 17.0).

RESULTS

A total of 200 cases 100 CAD and 100 non-CAD patients of age between 20-80 yrs were enrolled. The outcome measures of the study were demographic and clinical characteristics (age, sex, height, weight, BMI, educational status, nature of work, diet, smoking, alcohol, exercise, hypertension, and chest pain), biochemical parameters (HbA1c, HDL, LDL, VLDL, TG, TC, sdLDL, folate II, Vitamin B12, Vitamin D, TSH, total HCY2), markers (miR-122, miR-126 fold expression (ΔΔCt) and VEGF (pg/ml).

Demographic and clinical characteristics of CAD and non-CAD cases

The demographic and clinical characteristics of the two groups (non-CAD cases and CAD cases) are summarized in table 1. The age of non-CAD and CAD cases ranged from 25-79 yrs and 28-76 yrs with a mean (± SE) of 50.90 ± 2.08 yrs and 52.15 ± 1.13 yrs, respectively, and a median age of 52 yrs. Further, in non-CAD cases, there were 26 (26.0%) females and 74 (74.0%) males whereas in CAD cases, this was 25 (25.0%) and 75 (75.0%), respectively. Comparing the demographic and clinical characteristics of the two groups, χ2 test showed significantly different and higher frequency (%) of illiterate (14.0% vs. 61.0%, $\chi2=19.14$, $p<0.001$), smokers (16.0% vs. 37.0%, $\chi2=4.39$, $p=0.03$), those suffering from hypertension (24.0% vs. 62.0%, $\chi2=10.55$, $p=0.001$) and those from chest pain (17.0% vs. 71.0%, $\chi2=44.42$, $p<0.001$) whereas, less frequency of exercise (53.0% vs. 30.0%, $\chi2=4.73$, $p=0.03$) in CAD cases as compared to non-CAD suggests that these parameters may be associated with CAD. However, other demographic and clinical characteristics (age, sex, height, weight, BMI, occupation, nature of work, diet, and alcohol) were found to be similar ($p>0.05$) between the two groups indicating these parameters may not be associated with CAD development.

Values of biochemical parameters values in CAD and non-CAD cases

The level of biochemical parameters in the level of the two groups is summarized in table 2. Comparing the biochemical parameter levels of two groups, χ^2 test showed significantly different and higher level of (%) of HbA1c (10.0% vs. 41.0%, $\chi^2=9.52$, $p=0.002$), sdLDL (17.0% vs.

62.0%, χ^2=17.46, p<0.001), folate II (20.0% vs. 60.0%, χ^2=14.05, p<0.001), Vitamin B12 (27.0% vs. 59.0%, χ^2=9.05, p=0.003) and VEGF (pg/ml) (20.0% vs. 62.0%, χ^2=14.95, p<0.001) whereas, less frequency of HDL (63.0% vs. 43.0%, χ^2=3.84, p=0.05) and LDL (70.0% vs. 40.0%, χ^2=7.95, p=0.005) in CAD cases as compared to non CAD cases suggesting that these may be associated with CAD. However, biochemical parameters namely VLDL, TG, TC, and Vitamin D, TSH, and total HCY2 did not differ (p>0.05) between the two groups indicating that these may not be associated with CAD. We have also observed the correlation of miR-122 and -126 with demographic and clinical characteristics of the cases, however, the difference was not significant (data not shown).

Circulating miR-122 and miR -126 expression

The marker expression levels in the two groups are summarized in Table 3. Comparing the marker expression levels of the two groups, χ2 test showed significantly different and higher frequency (%) of miR-122 (14.0% vs. 63.0%, χ2=21.23, p<0.0001) whereas the significantly different and lower frequency of miR-126 (80.0% vs. 39.0%, χ2=14.95, p<0.0001) in CAD cases as compared to non-CAD suggests that both markers may be associated with CAD as depicted in figure 1a and 1b.

Independent predictors of CAD

Unadjusted odds ratio

To evaluate the risk predictors of CAD, all variables (demographic and clinical, biochemical and marker expression) were first subjected to univariate (crude or unadjusted) binary logistic regression analysis (controls=0 and cases=1). These are summarized in Table 4. The univariate analysis found educational status (OR=9.86, 95% CI=3.13-31.01, p<0.001), smoking (OR=3.02, 95% CI=1.04-8.76, p=0.04), exercise (OR=0.39, 95% CI=0.16-0.92, p=0.03), hypertension (OR=4.40, 95% CI=1.74-11.14, p=0.002), chest pain (OR=27.50, 95% CI=8.79-86.01, p<0.001), HbA1c (OR=6.26, 95% CI=1.75-22.41, p=0.005), LDL (OR=0.28, 95% CI=0.12-0.70, p=0.006), sdLDL (OR=8.00, 95% CI=2.76-23.16, p<0.001), folate II (OR=6.07, 95% CI=2.22-16.53, p<0.001), Vitamin B12 (OR=3.95, 95% CI=1.57-9.98, p=0.004), VEGF (OR=6.40, 95% CI=2.35-17.47,

Table 1 Demographic and clinical characteristics of two groups

Variable	Controls (n=100) (%)	Cases (n=100) (%)	χ2 value	p value
Age (yrs.):				
≤52	56 (56.0)	52 (52.0)	0.15	0.702
>52	44 (44.0)	48 (48.0)		
Sex:				
Female	26 (26.0)	25 (25.0)	0.01	0.913
Male	74 (74.0)	75 (75.0)		
Height (cm):				
≤165	53 (53.0)	69 (69.0)	2.40	0.121
>165	47 (47.0)	31 (31.0)		

Weight (kg): ≤65 >65	 60 (60.0) 40 (40.0)	 56 (56.0) 44 (44.0)	0.11	0.735
BMI (kg/m2): ≤26 >26	 67 (67.0) 33 (33.0)	 56 (56.0) 44 (44.0)	0.94	0.331
Educational status: Literate Illiterate	 86 (86.0) 14 (14.0)	 39 (39.0) 61 (61.0)	19.14	<0.001
Occupation: No Yes	 53 (53.0) 47 (47.0)	 34 (34.0) 66 (66.0)	3.17	0.075
Nature of work: Sedentary Hard	 22 (22.0) 78 (78.0)	 27 (22.0) 73 (73.0)	0.29	0.591
Diet: Vegetarian Non-vegetarian	 43 (43.0) 57 (57.0)	 27 (27.0) 73 (73.0)	0.43	0.513
Smoking: No Yes	 84 (84.0) 16 (16.0)	 63 (63.0) 37 (37.0)	4.39	0.036
Alcohol: No Yes	 83 (83.0) 17 (17.0)	 90 (90) 10 (10.0)	0.18	0.670
Exercise: No Yes	 67 (67.0) 53 (53.0)	 70 (70.0) 30 (30.0)	4.73	0.030
Hypertension: No Yes	 76 (76.0) 24 (24.0)	 38 (38.0) 62 (62.0)	10.55	0.001
Chest pain: No Yes	 83 (83.0) 17 (17.0)	 29 (29.0) 71 (71.0)	44.42	<0.001

Demographic and clinical characteristics of two groups were summarised in number (n) and percentage (%) and compared by $\chi 2$ test.

Table 2 Biochemical parameter levels of two groups

Variable	Controls (n=100) (%)	Cases (n=100) (%)	$\chi 2$ value	p value
HbA1c (%):				
≤6	90 (90.0)	59 (59.0)	9.52	0.002
>6	10 (10.0)	41 (41.0)		
HDL (mg/dl):				
≤43	37 (37.0)	57 (57.0)	3.84	0.05
>43	63 (63.0)	43 (43.0)		
LDL (mg/dl):				
≤56	30 (30.0)	60 (60.0)	7.95	0.005
>56	70 (70.0)	40 (40.0)		
VLDL (mg/dl):				
≤29	50 (50.0)	53 (53.0)	0.06	0.81
>29	50 (50.0)	47 (47.0)		
TG (mg/dl):				
≤133	57 (57.0)	47 (47.0)	0.74	0.39
>133	43 (43.0)	53 (53.0)		
TC (mg/dl):				
≤135	37 (37.0)	56 (56.0)	3.38	0.06
>135	63 (63.0)	44 (44.0)		
sdLDL (mmol/l):				
≤15	83 (83.0)	38 (38.0)	17.46	<0.001
>15	17 (17.0)	62 (62.0)		
Folate II (mmol/l):				
≤9	80 (80.0)	40 (40.0)	14.05	<0.001
>9	20 (20.0)	60 (60.0)		
Vitamin B12 (pg/ml):				
≤238	73 (73.0)	41 (41.0)	9.05	0.003
>238	27 (27.0)	59 (59.0)		
Vitamin D (ng/ml):				
≤21	40 (40.0)	60 (60.0)	3.59	0.05
>21	60 (60.0)	40 (40.0)		

TSH (µmol/l): ≤2 >2	 63 (63.0) 37 (37.0)	 64 (64.0) 36 (36.0)	0.01	0.94
Total HCY2 (µmol/l): ≤19 >19	 47 (47.0) 53 (53.0)	 52 (52.0) 48 (48.0)	0.30	0.58
VEGF (pg/ml): ≤120 >120	 80 (80.0) 20 (20.0)	 38 (38.0) 62 (62.0)	14.95	<0.001

Biochemical parameter levels of two groups were summarised in number (n) and percentage (%) and compared by χ2 test.

Table 3 Circulating miRNA 122 &126 expression levels of two groups

Variable	Cut off	Controls (n=100) (%)	Cases (n=100) (%)	χ2 value	p value
miR-122:	≤1.24 >1.24	86 (86.0) 14 (14.0)	37 (37.0) 63 (63.0)	21.23	<0.0001
miR-126:	≤0.90 >0.90	20 (20.0) 80 (80.0)	61 (61.0) 39 (39.0)	14.95	<0.0001

Expression levels of two groups were summarised in number (n) and percentage (%) and compared by χ2 test.

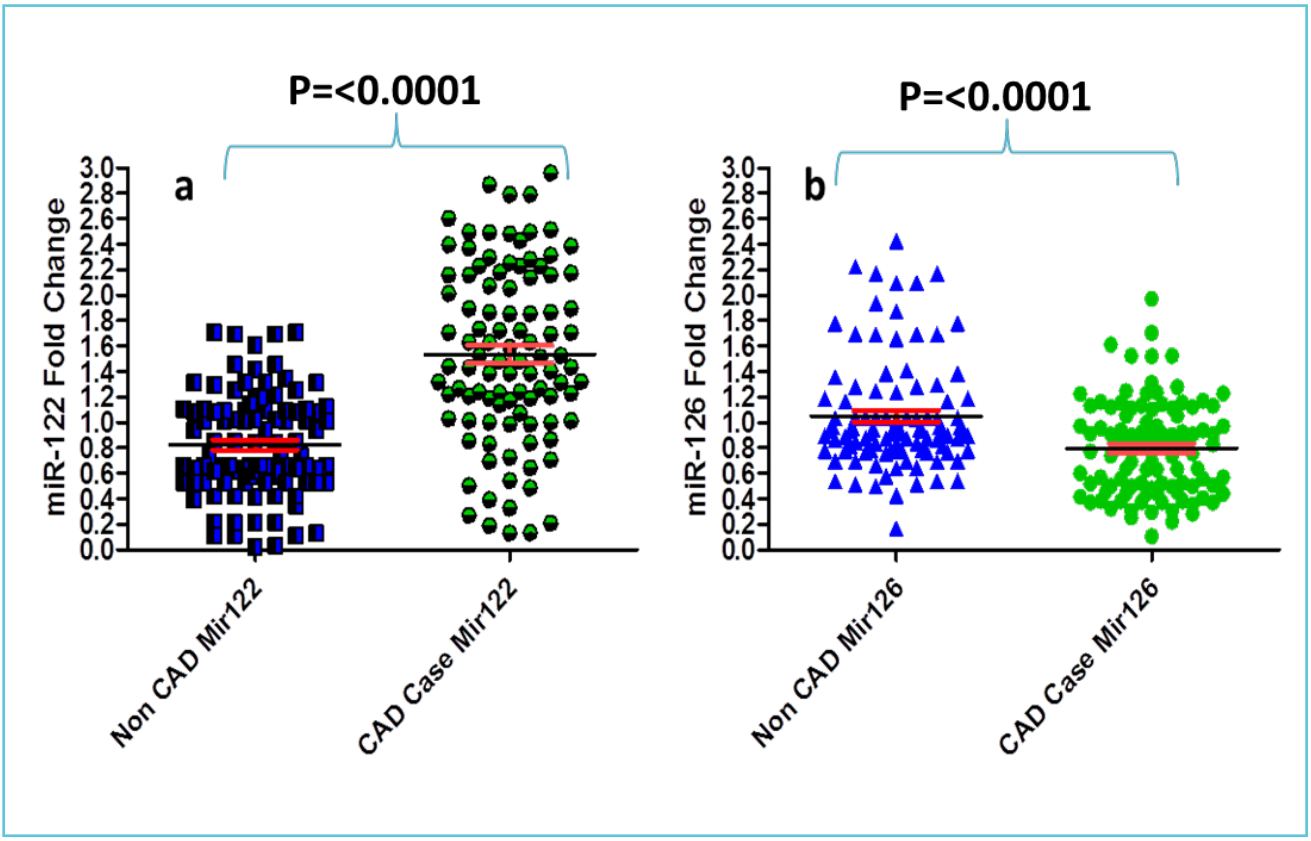
Table 4 Predictors of CAD using univariate binary logistic regression analysis

Predictor	OR (95% CI)	p value
Educational status: Literate Illiterate	 Ref. 9.86 (3.13-31.01)	 <0.001
Smoking: No Yes	 Ref. 3.02 (1.04-8.76)	 0.04

Exercise: No Yes	 Ref. 0.39 (0.16-0.92)	 0.03
Hypertension: No Yes	 Ref. 4.40 (1.74-11.14)	 0.002
Chest pain: No Yes	 Ref. 27.50 (8.79-86.01)	 <0.001
HbA1c (%): ≤6 >6	 Ref. 6.26 (1.75-22.41)	 0.005
LDL (mg/dl): ≤56 >56	 Ref. 0.28 (0.12-0.70)	 0.006
sdLDL (mmol/l): ≤15 >15	 Ref. 8.00 (2.76-23.16)	 <0.001
Folate II (mmol/l): ≤9 >9	 Ref. 6.07 (2.22-16.53)	 <0.001
Vit. B12 (pg/ml): ≤238 >238	 Ref. 3.95 (1.57-9.98)	 0.004
VEGF (pg/ml): ≤120 >120	 Ref. 6.40 (2.35-17.47)	 <0.001
miR-122: ≤1.24 >1.24	 Ref. 10.98 (3.48-34.63)	 <0.001
miR-126: ≤0.90 >0.90	 Ref. 0.16 (0.06-0.43)	 <0.001

***OR:** odds ratio, **CI:** confidence interval, **Ref:** reference category. All odds ratio were evaluated against reference category.*

Figure 1 Scatter plot showing values of the a) miR-122 and b) miR-126 in CAD cases and non-CAD cases



*Groups were compared by Man Whitney U test. (Nsp>0.05 or***p< 0.001- as compared to Control group.)*

p<0.001), miR-122 (OR=10.98, 95% CI=3.48-34.63, p<0.001) and miR-126 (OR=0.16, 95% CI=0.06-0.43, p<0.001) as the significant predictors of CAD. Predictors that have shown significant p values in univariate (crude or unadjusted) binary logistic regression analysis were subjected to multivariable repression model to find the independent risk predictors of CAD.

Adjusted odds ratio

To find out the independent risk predictors of CAD, the significant variables (found in Table 4) were further subjected to multivariate (adjusted) binary logistic regression analysis and are summarized in Table 5. The multivariate analysis further found chest pain (OR=37.07, 95% CI=3.21-169.04, p=0.01) and miR-126 (OR=0.01, 95% CI=0.00-0.63, p=0.03) as significant. Thus, chest pain and miR-126 may serve as significant and independent predictors of CAD.

Diagnostic accuracy of circulatory miR-122 and miR-126

To see the diagnostic accuracy (sensitivity and specificity) of markers (miR-122 and miR-126) in predicting CAD, both the markers were subjected to ROC curve analysis. The ROC curve analysis showed a significant diagnostic accuracy of miR-122 area under the curve (AUC= 0.8057, Z=6.66, p<0.001) and a cut-off value of >1.24, it discriminated the subjects of the two groups (controls and cases) with 64.00% sensitivity (95% CI=53.79-73.36) and 84.00% specificity (95% CI=75.32-90.57), and

79.75% positive predictive value and 69.42% negative predictive value. Similarly, miR-126 also showed significant diagnostic accuracy (AUC=0.806, Z=3.52, p<0.001), and at a cut-off value of ≤0.90, it discriminated the subjects of the two groups with 61.54% sensitivity (95% CI=49.8-72.3) and 80.00% specificity (95% CI=61.4-92.2), and 88.9% positive predictive value and 44.4% negative predictive value and also depicted in figure 2.

DISCUSSION

The role of circulating miRNAs as a new biomarker in diagnosing cardiovascular diseases has kindled a brighter outlook worldwide. Despite recent advances and a stupendous rise in interest in miRNA for CAD diagnosis and prognosis, we are still unraveling the complexity superficially. Circulating miRNAs are a sensitive, diagnostic, and prognostic biomarker for therapeutic interventions. MiRNAs show

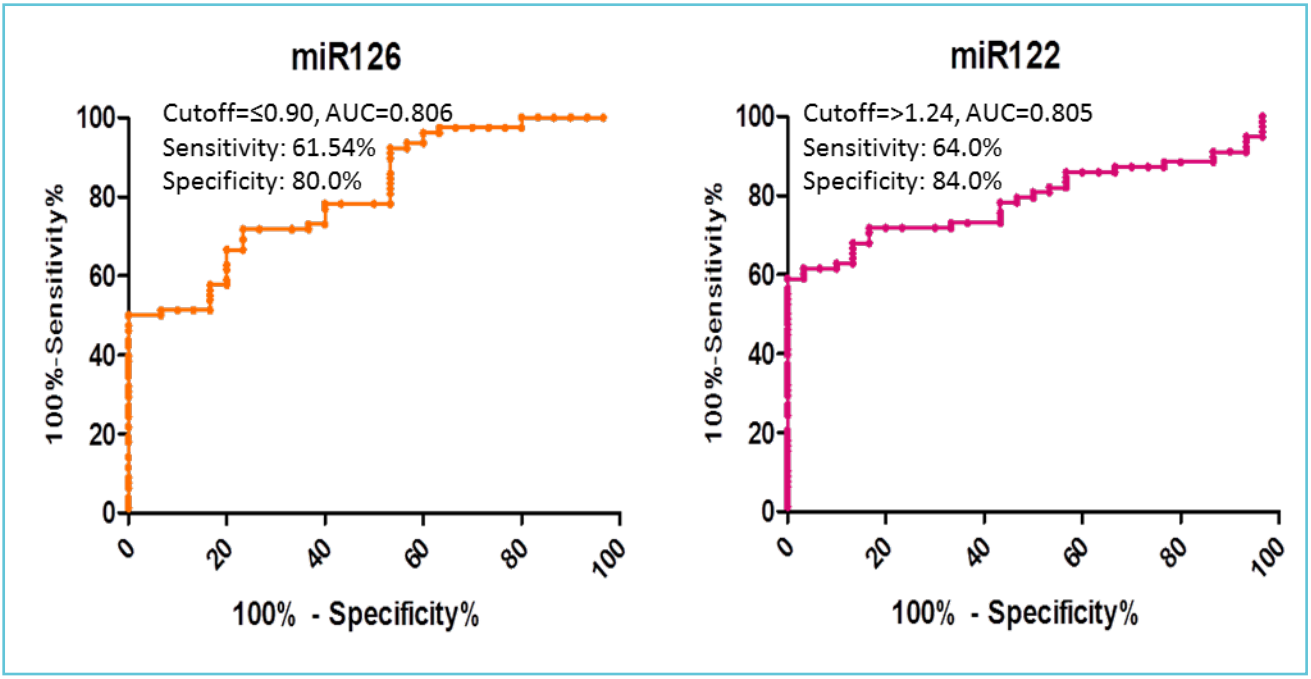
Table 5 Independent predictors of CAD using multivariate binary logistic regression analysis

Predictor	OR (95% CI)	p value
Educational status:		
Literate	Ref.	
Illiterate	4.94 (0.32-76.49)	0.25
Smoking:		
No	Ref.	
Yes	13.76 (0.54-353.17)	0.11
Exercise:		
No	Ref.	
Yes	0.12 (0.00-5.31)	0.27
Hypertension:		
No	Ref.	
Yes	1.15 (0.08-16.36)	0.91
Chest pain:		
No	Ref.	
Yes	6.60 (3.21-169.04)	0.01
HbA1c (%):		
≤6	Ref.	
>6	3.32 (0.16-68.21)	0.43
LDL (mg/dl):		
≤56	Ref.	
>56	0.15 (0.01-2.23)	0.16

sdLDL (mmol/l): ≤15 >15	 Ref. 18.20 (0.62-534.65)	 0.09
Folate II (mmol/l): ≤9 >9	 Ref. 6.34 (0.44-90.60)	 0.17
Vitamin B12 (pg/ml): ≤238 >238	 Ref. 26.62 (0.49-1454.89)	 0.10
VEGF: (pg/ml) ≤120 >120	 Ref. 1.31 (0.11-15.18)	 0.82
miR-122: ≤1.24 >1.24	 Ref. 13.25 (0.68-259.03)	 0.08
miR-126: ≤0.90 >0.90	 Ref. 0.01 (0.00-0.63)	 0.03

***OR**: odds ratio, CI: confidence interval, **Ref:** reference category. All odds ratio were evaluated against reference category*

Figure 2 **ROC curve values for miR-126 and miR-122 to differentiate between CAD cases and non-CAD cases**



tissue-specific expression, are rapidly released into circulation, and are remarkably stable in serum/plasma. The emerging role of miRNA in CAD and cardiac arrhythmia has been demonstrated.[14] Analysis of circulating plasma/serum miRNA may be of importance in disease prediction and improvement of the diagnostic accuracy of cardiovascular diseases.[15, 16] Circulating levels of miR-1, miR-126, miR-133, miR-1291, miR-663b, and miR423-5p have been reported to be promising biomarkers in acute myocardial infarction (AMI) cases.[17, 18] Studies reported the up-regulation of miR-208a, miR-133a, miR-1, miR-133b, miR-337-5p, miR-122, and miR-433 in CAD cases, however, the circulating levels of miR-126, miR-17, miR-145, miR-155, miR-92a, and miR-199a were significantly down regulated in CAD cases, as compared to non-CAD cases (controls).[19] Hoeckstra et al. in 2010, for the first time, analyzed human circulating miR in CAD, and miR-135a and -147 were found to be significantly elevated in CAD cases compared to non-CAD healthy controls.[20] The findings highlight the role of circulating miRNAs as biomarkers in the detection of CAD. Different miRNAs related to cardiac origin have been studied to investigate the diagnostic potential. Reduced expression of miR-122 has been found in patients with MI. [21, 22] Our study evaluated the potential signature of circulating miRNAs 122 and 126 in differentiating CAD from non-CAD cases. We have found significant downregulation of miR-126 (p=<0.001) in CAD cases as compared to non-CAD cases. The level of circulating miR-122 was significantly upregulated in CAD cases compared to non-CAD cases (p=0.001). A study by Wang X et al. 2017, reported a significant down regulation of miR-126 in CAD cases, and it was also associated with increased placental growth factor (PGF) levels in CAD and AMI cases.[23] Our findings of miR-126 are consistent with those of Li HY et al. 2016, where plasma miR-126-5p was significantly downregulated in patients with severe CAD.[24] The levels of miR-126 were also lower in CAD patients with either intermediate or high SYNTAX scores, instead of low SYNTAX scores. In contrast to our finding, the study by Sridhar M et al. 2021 reported that miR-122 was significantly downregulated in CAD patients, however, the level of miR-126 did not show any change. This may be due to the frequent use of aspirin and β blockers medication of the study participants that may affect the miR-126 level.[13] These findings collectively indicate that anti-platelet therapies did not affect the miRNA levels. [25-28]

In our study, a univariate analysis of demographic, clinical characteristics and miR-126 & 122 has been carried out to find the risk predictor of CAD. We have found educational status (p<0.001), smoking (p=0.042), exercise (p=0.032), hypertension (p=0.002), chest pain (p<0.001), HbA1c (p=0.005), LDL (p=0.006), sdLDL (p<0.001), Folate II (p<0.001), Vitamin B12 (p=0.004), VEGF (p<0.001), miR-122 (p<0.001) and miR-126 (p<0.001) as a significant predictors of CAD. However, on multivariate analysis chest pain (p=0.017) and miR-126 (p=0.030) were found as a significant independent risk predictor of CAD. A study by Su T et al. 2019; reported that circulating miR-1 might be an important biomarker for early AMI diagnosis and may predict the prognosis of patients with chest pain.[29] Similarly, Fichtlscherer et al. in their study reported significantly reduced levels of endothelial expressed miR-126, -92a, and -17 in CAD cases compared to non-CAD cases.[30] A prospective population-based cohort study by Zampetaki et al. 2012 evaluated the predictive value of miRNA concerning myocardial infarction (MI). Multivariate cox regression analysis of miR-126, -197, and -223 showed predictive value for MI. The expression level of miR-126 was found to be positively associated with MI prediction while those of miR-197 and -223 were inversely associated with MI prediction.[31] A study

by Zhu L 2017; reported that the presence of circulating miRNA-133a level in blood was a risk factor of CHD (OR: 2.565, 95% CI: 1.105-5.954, P = 0.028) and showed a positive correlation with miR-133a expression and Gensini score in patients with CHD (r = 0.303, P = 0.007).[32] In a recent study, Gao W et al. 2012 showed that plasma levels of miR-122 significantly increased in hyperlipidemic patients as compared to controls and were positively correlated with TC, TG, and LDL-C levels in both hyperlipidemic patients and controls. Multiple logistic regression analyses revealed the presence of CAD in patients with increased levels of miR-122.[33] A study by Wang YL et al. 2018 showed the significantly upregulated level of miR-122 in patients with atherosclerotic lesions and these were positively correlated with cholesterol, triglycerides, and atherosclerotic severity.[34] A study by Pilbrow et al. 2014 reported that miR-652 was an independent predictor of heart failure after acute myocardial infarction (AMI).[35] However, the use of a combination of miRNAs or a combination of miRNAs with established prognostic markers such as BNP or cardiac troponin seems to improve the risk management in cardiovascular diseases.[35. 36] In our study, the HbA1c level was significantly different between the CAD cases and non CAD cases indicating that patients with diabetes mellitus may affect the extracellular miRNA expressions. [37, 27, 28]

Several studies have evaluated the diagnostic values of circulating miRNAs and reported that miRNA values may have diagnostic potential in the identification and differentiation of coronary artery disease.[34, 35, 36] In a study by Zhong Z et al. 2018, analyzed miRNA-126-5p in unstable angina (UA) patients and ROC analysis revealed an AUC of 0.714 (95% CI: 0.555–0.873), however for ST-segment elevation myocardial infarction (STEMI) patients AUC was 0.703 (95% CI: 0.541–0.864).[38] In our study, ROC analysis of miR-122 and miR-126 showed significant diagnostic accuracy with an AUC of 0.805 and 0.806 respectively..

These results strongly indicate that serum miR-126 and miR-122 might be used as a novel, non-invasive, and risk predictive biomarkers for CAD patients. Likewise, many studies have found the association of miRNAs with restenosis-related processes, such as VSMC proliferation, migration, and neointima formation highlighting the great use for diagnosis, prognosis, therapeutic, or in additional clinical management. [39-41] Present study advocates the use of circulating miRNAs in the field of population-based risk assessment of CAD. However, to fully exploit the potential of miRNAs, there is a need to standardized, sample processing as well as to use advanced techniques for analysis. Our study is limited by a smaller sample size. Therefore, future studies on larger cohorts without CAD (CAD-ve) and patients with CAD (CAD +ve) are needed to broadly evaluate the miRNAs for risk prediction in comparison with other established cardiac markers. Our findings suggest that blood-based miRNAs may be sensitive and specific biomarkers for monitoring cardiovascular diseases, and for the evaluation of myocardial protection during cardiac surgery. Further, an in-depth evaluation of the role of these miRNAs in the pathogenesis and progression of CAD will contribute to our understanding of the disease process and lead to new therapeutic and preventive strategies.

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Abbreviations used:

BNP - Brain Natriuretic Peptide
CAD - Coronary Artery disease
Echo - Echocardiogram
CT - Computed Tomography
ECG - Electrocardiogram
HDL - High density lipoproteins
HF - Heart failure

LDL - Low density lipoproteins
miRNA - MicroRNA
MI - myocardial infarction
TG - Triglyceride
VLDL -Very low-density lipoproteins
sdLDL - Small dense low-density lipoproteins
VEGF - Vascular Endothelial Growth Factor

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A combined analysis of serum Growth Differentiation Factor-15 and Cancer Antigen 15-3 enhances the diagnostic efficiency in breast cancer

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ABSTRACT

Introduction

Existing diagnostic biomarkers of breast cancer (BC) are limited by poor sensitivity. In this study, we evaluated the role of serum GDF-15 in early BC diagnosis, independently and in combination with CA15-3, a known blood biomarker of BC.

Material and methods

A total of 113 diagnosed, pre-therapy BC patients and 54 healthy controls were recruited. Clinical characteristics, TNM staging, and hormone receptor status of the patients were recorded. Serum GDF-15 and serum CA15-3 were measured by sandwich ELISA and chemiluminescence assay, respectively.

Results

The serum GDF-15 levels were significantly ($p<0.001$) elevated in BC patients compared to healthy controls and in patients with larger tumor size, advanced disease stage, and distant metastasis. ROC analysis revealed that at the cut-off of 525.77 pg/mL, GDF-15 had greater sensitivity than CA15-3. GDF-15 and CA15-3 performed better in combination than individually, with the combined test having an AUC of 0.85 and sensitivity and specificity of 0.63 and 0.98, respectively.

Further, serum GDF-15 had a better predictive ability for early-stage BC compared to CA15-3. GDF-15 could independently diagnose BC patients after adjusting for age.

Conclusion

We conclude that serum GDF-15 is a promising, robust marker for detecting early-stage BC. However, larger prospective studies are necessary to validate this claim.

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Abbreviations:

APE: Apurinic/apyrimidinic endonuclease
BC: Breast cancer
CA15-3: Cancer antigen 15-3
CEA: Carcinoembryonic antigen
EGFR: Epidermal growth factor receptor
GDF-15: Growth Differentiation Factor-15
MIC-1: Macrophage inhibitory cytokine-1
NSE: Neuron-specific enolase
TGF-β: Transforming growth factor-β

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INTRODUCTION

Breast cancer (BC), a heterogeneous disease, has differentially expressed cell surface receptors.

It is the commonest cancer in females (11.6% of all cancers), causing 626,679 deaths worldwide, making it the second leading cause of cancer mortality (1). Early diagnosis is crucial to prevent poor outcomes. Currently, BC diagnosis relies upon mammography, but it has limited sensitivity (2,3). Some blood-based biomarkers can detect cancer early on before the appearance of clinical symptoms, e.g. CA125 for ovarian cancer (4). At present, blood-based biomarkers of BC such as CA15-3 and BR27.29 are not reliable due to their low sensitivity (5). Thus, there is an unmet need for early diagnostic biomarkers that could predict disease outcomes and prevent the poor prognosis of BC.

CA15-3 concentrations are elevated in BC as well as in pancreatic, ovarian, lung, colon and liver cancers (6,7). Nieder et al. 2017 (8) have observed that it can be used as a prognostic marker for monitoring therapeutic response as elevated levels increased with tumour size and disease progression. But higher values have been reported in benign conditions, implying low sensitivity (6). A meta-analysis conducted by Fu et al. (9) showed that elevated CA15-3 was significantly associated with malignant breast tumours.

GDF-15, also called macrophage inhibitory cytokine-1 (MIC-1), is a member of the transforming growth factor-β (TGF-β) superfamily. It has a molecular weight of 25-kDa. GDF-15 is vital in cancer proliferation, apoptosis, migration, angiogenesis, and immune modulation (10). An increase in serum GDF-15 is associated with pathological grade, staging, lymph node involvement, and other clinical outcomes and prognosis of multiple cancers such as ovarian, hepatocellular, prostate and lung cancer

(10-15). Furthermore, some studies reported that an increase in GDF-15 expression is associated with proliferation, migration, invasion and stemness of BC (16-18). However, the role of GDF-15 in BC diagnosis is unexplored.

The current study proposes to evaluate serum GDF-15 and CA15-3 as a diagnostic markers with clinicopathological features of BC. Serum GDF-15 could significantly delineate early-stage BC patients from healthy controls; diagnostic accuracy analysis further showed that it could be used as an early diagnostic marker, with high sensitivity and specificity, which improved in combination with CA15-3. Serum GDF-15 was also significantly higher in metastatic compared to non-metastatic BC.

MATERIALS & METHODS

2.1 Ethics statement

The study was carried out in compliance with the ethical principles for medical research involving human subjects, in accordance with the declaration of Helsinki. Ethical approval was granted from the Institutional Ethics Committee (IEC) of AIIMS, Jodhpur. Written informed consent was taken from each participant before enrolling in the study.

2.2 Study population

Sera from 113 BC patients were collected between August 2018 and March 2020 from the outpatient clinics of the Department of Radiation Oncology and the Department of Surgical Oncology at AIIMS, Jodhpur. 54 healthy subjects were recruited by a physical examination. The inclusion criteria for BC patients were 1) aged >18 years, 2) primary BC patients who had not received any chemotherapy, i.e. newly diagnosed BC without prior treatment, 3) diagnosed through mammography and histopathological examination. Participants were ineligible if they were histologically diagnosed with other conditions such as benign tumour upon pathological review, prior history of any other malignancy, anti-cancer medication or were under palliative care. All the clinicopathological characteristics of BC, including TNM staging and IHC receptor status, were acquired from the hospital medical records only after taking consent from the patients.

2.3 Sample preparation

Blood samples for serum CA15-3 and GDF-15 were collected by venipuncture in clot activator vacutainer for serum separation. The serum was separated immediately by centrifugation and stored at -80°C. Sera were only thawed once, just before the analysis. Serum GDF-15 was measured in batches using GDF15 Human ELISA Kit purchased from Invitrogen, (Thermo Fisher Scientific, #EHGDF15) having a 2 pg/mL detection limit and coefficient of variation (CV) of <10% (intra-assay) and <12% (inter-assay) following the manufacturer's protocol. Serum CA15-3 was detected by chemiluminescent enzyme immunoassay on Diasorin Liaison.

2.4 Statistical analysis

Data were analyzed in SPSS version 22.0 and R (version 3.5.3) using RStudio (19). Continuous variables were expressed as median and interquartile range (IQR). Since serum GDF-15 and CA15-3 were not normally distributed, non-parametric tests were employed viz. Mann Whitney U for two groups and Kruskal Wallis Rank Sum test for three or more groups. Multiple comparisons (Dunn Test) were carried out if significance was found in more than two groups. To evaluate the diagnostic ability of GDF-15 and to merge diagnostic information of the predictors, Receiver Operating Characteristic (ROC) curve and binary logistic regression were used. For all analytical purposes, a two-tailed $p<0.05$ was considered to be statistically significant.

RESULTS

3.1 Clinico-pathological characteristics

A total of 113 BC patients were enrolled in this study which included 111 females and two males. Table 1 summarizes the somatometric characteristics of the patient population. The median age of the BC patients was 51 (IQR 19.5) years. 24 (21.24%) had comorbidities such as thyroid dysfunction, diabetes mellitus, hypertension and asthma among these patients. 88 (77.88%) patients did not have any comorbidities. There were 12 (10.62%) diabetics. Based on their menstruation history, 38 (34.23%) were pre-menopausal, and 73 (65.77%) were post-menopausal. The patients were staged as follows: 35 (30.97%) were early-stage (stages I-II), 77 (68.14%) were of advanced stage (stages III-IV). In these patients, 85 (75.22%) were non-metastatic and 27 (23.89%) were metastatic (Table 2).

Table 1 Somatometric variables of the patient population according to serum GDF-15 and CA15-3

Variables	n=113	GDF-15 (pg/mL)			CA15-3 (U/mL)		
		Median	IQR	*p*-value	Median	IQR	*p*-value
Age							
<50 years	47	537.70	513.32	0.012*	24.01	35.01	0.313
≥50 years	66	706.40	686.06		20.17	28.13	
Menstruation status							
Pre-menopausal	38	603.70	589.95	0.354	26.82	40.51	0.027*
Post-menopausal	75	658.67	640.11		18.89	24.60	
Comorbidity							
Present	24	851.21	907.18	0.110	22.96	54.13	0.420
Absent	88	603.70	479.22		21.03	21.99	
Diabetes Mellitus							
Present	12	1363.80	1338.10	0.001*	39.33	151.71	0.333
Absent	100	593.16	489.61		21.60	5.31	

** Statistically significant at p<0.05 after comparison by Mann Whitney U test.*
IQR: Interquartile range.

Table 2 Tumor characteristics according to serum GDF-15 and CA15-3

Variables	n=113	GDF-15 (pg/mL)			CA15-3 (U/mL)		
		Median	IQR	*p*-value	Median	IQR	*p*-value
Tumor size							
T1-T2	37	558.65	348.77	0.018*	16.42	14.51	<0.001*
T3-T4	75	707.12	701.59		28.27	41.27	
Nodal status							
N0	48	618.47	495.28	0.384	18.60	13.09	0.031*
N1-3	64	627.10	710.20		28.27	41.14	
Metastasis							
M0	85	603.70	457.51	0.014*	19.07	19.16	0.001*
M1	27	927.49	1461.40		36.26	177.85	
Staging							
Early	35	551.03	322.01	0.006*	15.20	14.24	<0.001*
Advanced	77	707.12	807.73		28.27	39.68	
IHC receptor status							
ER+	53	659.81	840.30	0.266	20.43	23.52	0.888
ER-	35	634.40	363.78		21.21	25.00	
PR+	33	884.54	1146.90	0.059	21.29	23.58	0.772
PR-	55	629.95	445.30		20.22	22.40	
Her2+	33	707.80	663.15	0.739	27.84	23.96	0.103
Her2-	55	613.02	469.43		19.56	22.12	

TNBC status							
TNBC	23	604.89	315.84	0.221	22.16	25.61	0.792
Non-TNBC	65	706.40	646.70		20.17	22.62	

** Statistically significant at p<0.05 after comparison by Mann Whitney U test.*
IHC: Immunohistochemistry, IQR: Interquartile range.

3.2 Serum GDF-15 and CA15-3 were elevated in breast cancer

We detected increased levels of serum GDF15 (median [IQR] 625.46 [530.94] pg/mL) in BC patients compared with healthy subjects (median [IQR] 385.31 [202.57] pg/mL; p<0.001) (Figure 1). Moreover, when all patients with BC were grouped according to TNM classification, the level of serum GDF-15 gradually increased with the staging of BC (p<0.001) (Figure 1); also, serum GDF-15 levels were significantly higher in the early-stage group (stage I-II) (median [IQR] 551.03 [322.01] pg/mL) in comparison with healthy controls (median [IQR] 385.31 [202.57] pg/mL; p=0.009), suggesting that an elevated serum GDF15 might present in the early stage of BC. There was a gradual increment in serum GDF-15 levels, with higher levels in advanced patients (Stage III-IV) (median [IQR] 707.12 [807.73] pg/mL; p=0.006) compared with early-stage (I-II) patients, implying the positive correlation of GDF-15 with BC progression.

Further analysis showed that the level of serum GDF-15 was higher in BC patients with larger tumour size (T3-T4) (p=0.018), progesterone receptor (PR) positive status (p=0.059), and distant metastasis (M1) (p=0.014) compared to small tumour size (T1-T2), PR negative status or in the absence of distant metastasis (M0), respectively (Table 2). Serum GDF-15 level was also significantly higher in patients with diabetes (p=0.001). Additionally, data also indicated a significant association between the level of serum GDF-15 and age (p<0.001); with advancing age, an increasing trend was observed. However, no statistical association of serum GDF-15 with nodal involvement, menstruation or receptor status was reported.

Likewise, we observed that serum CA15-3 concentration was significantly higher in BC patients (median [IQR] 21.640 [26.51] U/mL) than in healthy controls (median [IQR] 13.614 [9.37] U/mL; p<0.001). Both early-stage (median [IQR] 15.200 [14.24] U/mL; p=0.316) and advanced-stage patients (median [IQR] 28.275 [39.68] U/mL; p<0.001) showed elevated CA15-3 levels (Figure 2), but the increase in early-stage was statistically not significant, unlike serum GDF-15. CA15-3 was also significantly higher in BC patients with larger tumor size (T3-T4) (p<0.001), nodal involvement (p=0.031), metastasis (p=0.001), advanced-stage (p<0.001), and post-menopausal status (p=0.027) (Table 1 and Table 2).

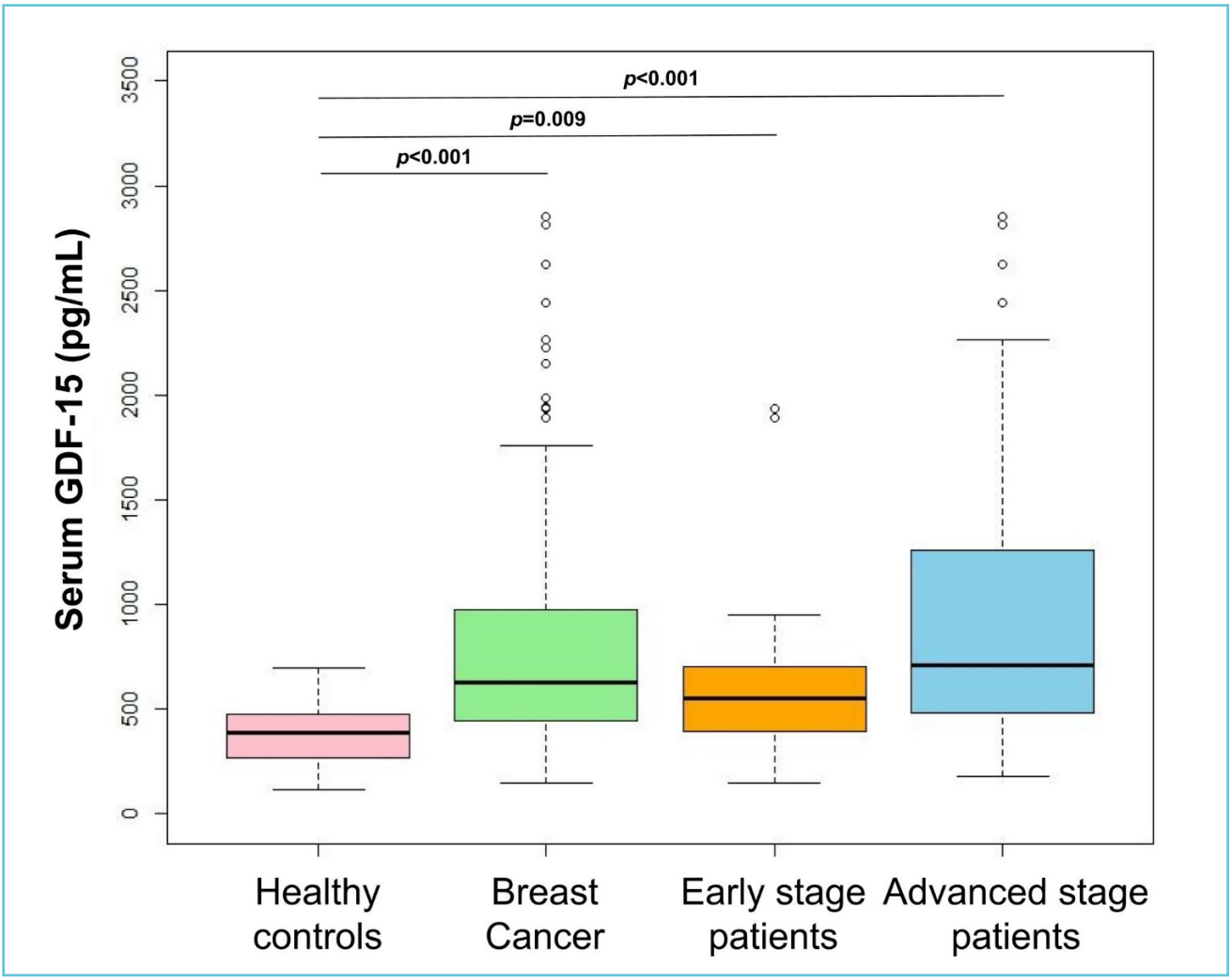
3.3 Diagnostic performance of serum GDF-15 compared to CA 15-3

Our study assessed serum GDF-15 as a non-invasive, diagnostic biomarker for BC compared to CA15-3 by ROC curve analysis. Using 54 normal samples as controls, the calculated AUC of GDF-15 was 0.790 (Figure 3A), compared to CA15-3, which had an AUC of 0.747 (Table 3, Figure 3B). To establish serum GDF-15 as a marker for BC, we used the *cutpointr* package to calculate optimal cut-off values. We found the serum GDF-15 cut-point for our study population to be 525.77 pg/ml by maximizing the sum of sensitivity and

specificity. The Youden's Index of GDF-15 in the diagnosis of BC was 0.528 at the 525.77 pg/ml, with a sensitivity and specificity of 65.77% and 87.04%, respectively and an accuracy of 72.73%. Also, CA15-3 had a sensitivity, specificity, and accuracy of 47.75%, 92.59%, and 62.42%, respectively. The combination of both GDF-15 and CA15-3 had an improved AUC of 0.846, with a sensitivity and specificity of 63.06% and 98.15%, respectively. Further, the combined AUC was significantly better than CA15-3 alone (p=0.003 by DeLong's test for comparison of two correlated empirical ROC curves) (Figure 3C).

In early-stage BC (n=34), GDF-15 had a sensitivity and specificity of 73.53% and 68.52%, respectively, with an AUC of 0.726 (p<0.001) (Table 3, Figure 4A). The serum GDF-15 cut-off point for early-stage BC was 426.35 pg/mL.

Figure 1 **Comparison of serum GDF-15 concentrations in breast cancer patients compared to healthy controls**



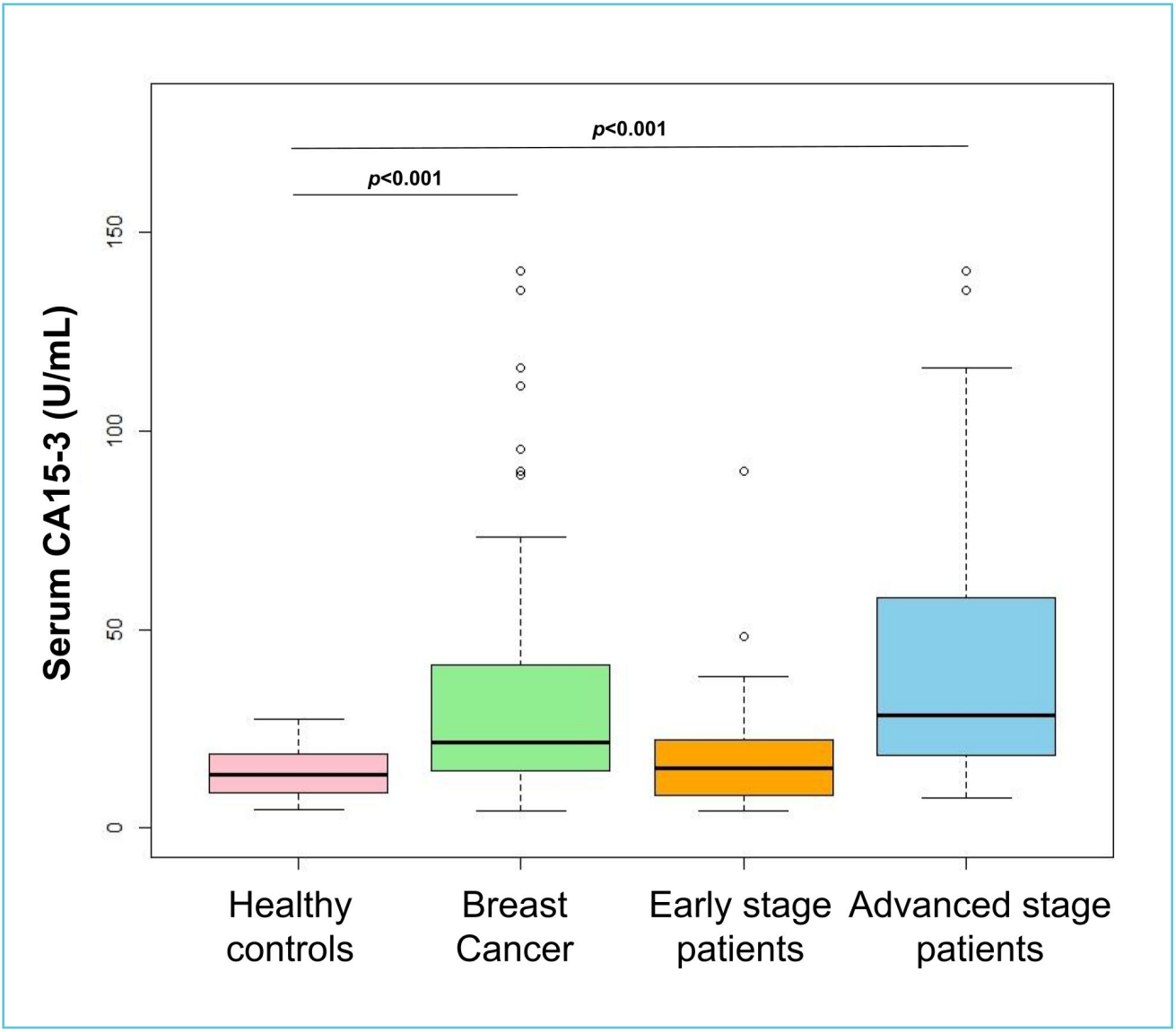
The breast cancer population is further grouped into early-stage and advanced-stage, and compared with the healthy control group. Between group comparison by Kruskal Wallis rank sum test revealed a highly significant difference (p<0.001). Further, a pairwise comparison by Dunn test with Holm correction showed that serum GDF-15 was significantly higher in breast cancer (adjusted p<0.001), early-stage breast cancer (adjusted p=0.009) and advanced-stage breast cancer (adjusted p<0.001) compared to healthy controls.

Serum CA15-3 had a specificity of 90.74% for early-stage BC with an AUC of 0.562 (p=0.326) (Figure 4B). Furthermore, the sensitivity and specificity of a combined GDF-15 and CA15-3 were 67.65% and 72.22%, respectively (AUC 0.734, p<0.001, Figure 4C), implying that serum GDF-15 overall is a better predictive marker for early-stage BC than CA15-3.

In metastatic BC patients, serum GDF-15 was found to have a sensitivity, specificity, and AUC of 50.00%, 82.14%, and 0.660, respectively (p=0.014) (Table 3).

In comparison, CA15-3 had better sensitivity (80.77%, AUC 0.720, p=0.001) than GDF-15 and the combination of GDF-15 and CA15-3 (50.00%, AUC 0.685, p=0.004).

Figure 2 **Comparison of serum CA15-3 levels in breast cancer patients in comparison to healthy controls**

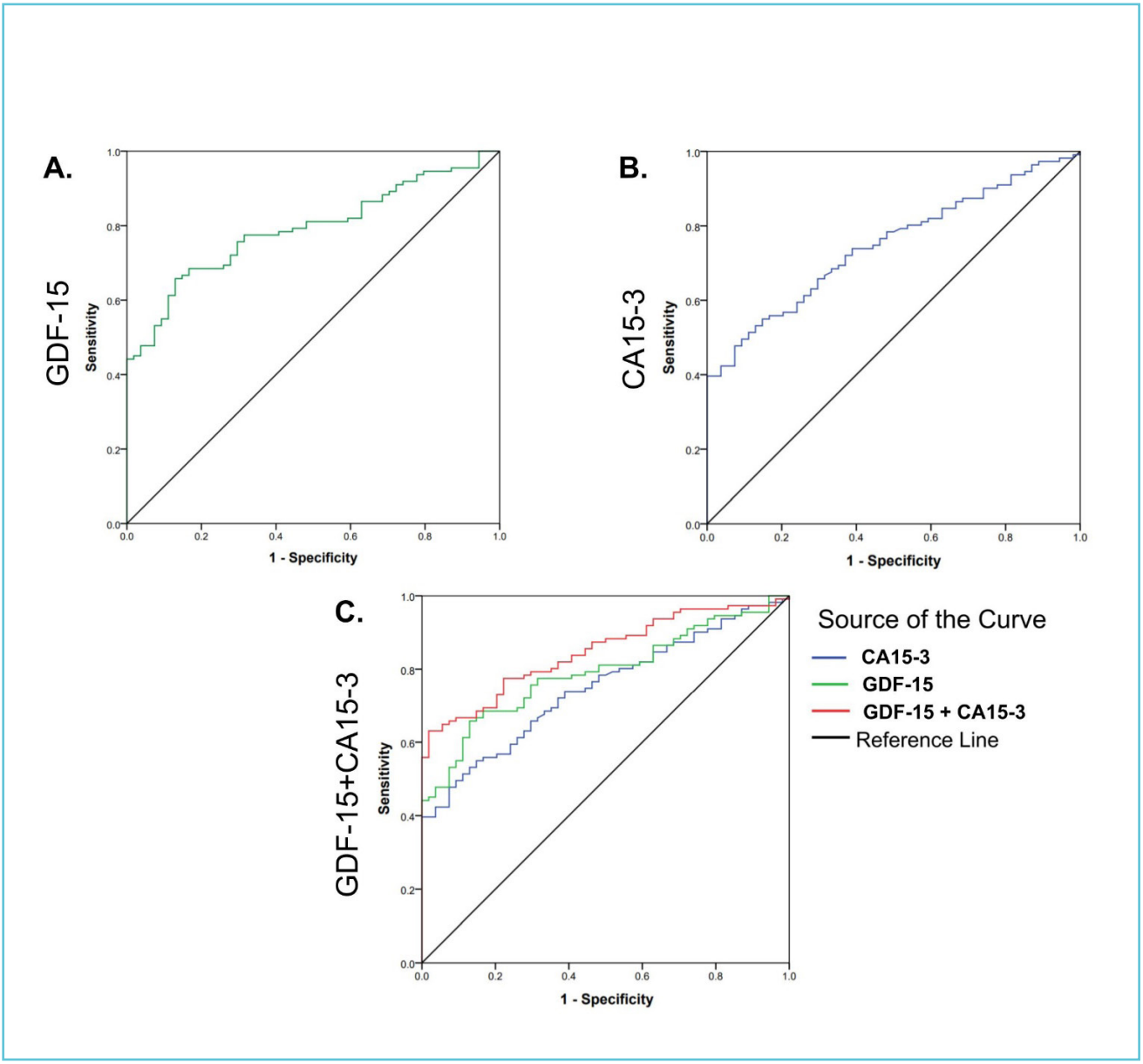


Between group comparison by Kruskal Wallis rank sum test revealed a highly significant difference (p<0.001). Multiple comparison by Dunn test with Holm correction showed that serum GDF-15 was significantly higher in breast cancer (adjusted p<0.001) and advanced-stage breast cancer (adjusted p<0.001) compared to healthy controls.

3.4 Serum GDF-15 is an independent predictor of BC patients

We further carried out a binary logistic regression, which showed serum GDF-15 as an independent predictor that could differentiate BC patients from controls after adjusting for age (Table 4). The adjusted serum GDF-15 had an OR of 5.76 (95% CI 1.98-16.79, p=0.001).

Figure 3 **Receiver Operating Characteristic (ROC) curve analysis for A. Serum GDF-15, B. Serum CA15-3, and C. the combination of both markers in breast cancer.**



The combined ROC curve has been plotted from the combined probabilities derived from logistic regression of serum GDF-15 and serum CA15-3 in the study population. The reference line signifies an AUC of 0.50. The combined markers had a sensitivity of 63.06% and a specificity of 98.15%, and the AUC (0.846) was significantly higher compared to serum CA15-3 alone (AUC 0.747, p=0.003).

Table 3 Area Under the Curve for serum GDF-15 and CA15-3 in breast cancer and early-stage breast cancer patients

Patient population	Marker	AUC	Std. Error	95% CI	*p*-value
Breast Cancer	GDF-15	0.790	0.034	0.723-0.857	<0.001*
	CA15-3	0.747	0.037	0.674-0.820	<0.001*
	GDF-15+CA15-3	0.846	0.029	0.789-0.903	<0.001*
Early-stage breast cancer	GDF-15	0.726	0.058	0.612-0.840	<0.001*
	CA15-3	0.562	0.066	0.434-0.691	0.326
	GDF-15+CA15-3	0.734	0.058	0.621-0.847	<0.001*
Metastatic breast cancer	GDF-15	0.660	0.067	0.529-0.792	0.014*
	CA15-3	0.720	0.059	0.604-0.836	0.001*
	GDF-15+CA15-3	0.685	0.068	0.551-0.819	0.004*

** Statistically significant at p<0.05*
Null hypothesis: true area = 0.50
Std. error has been calculated under the nonparametric assumption.

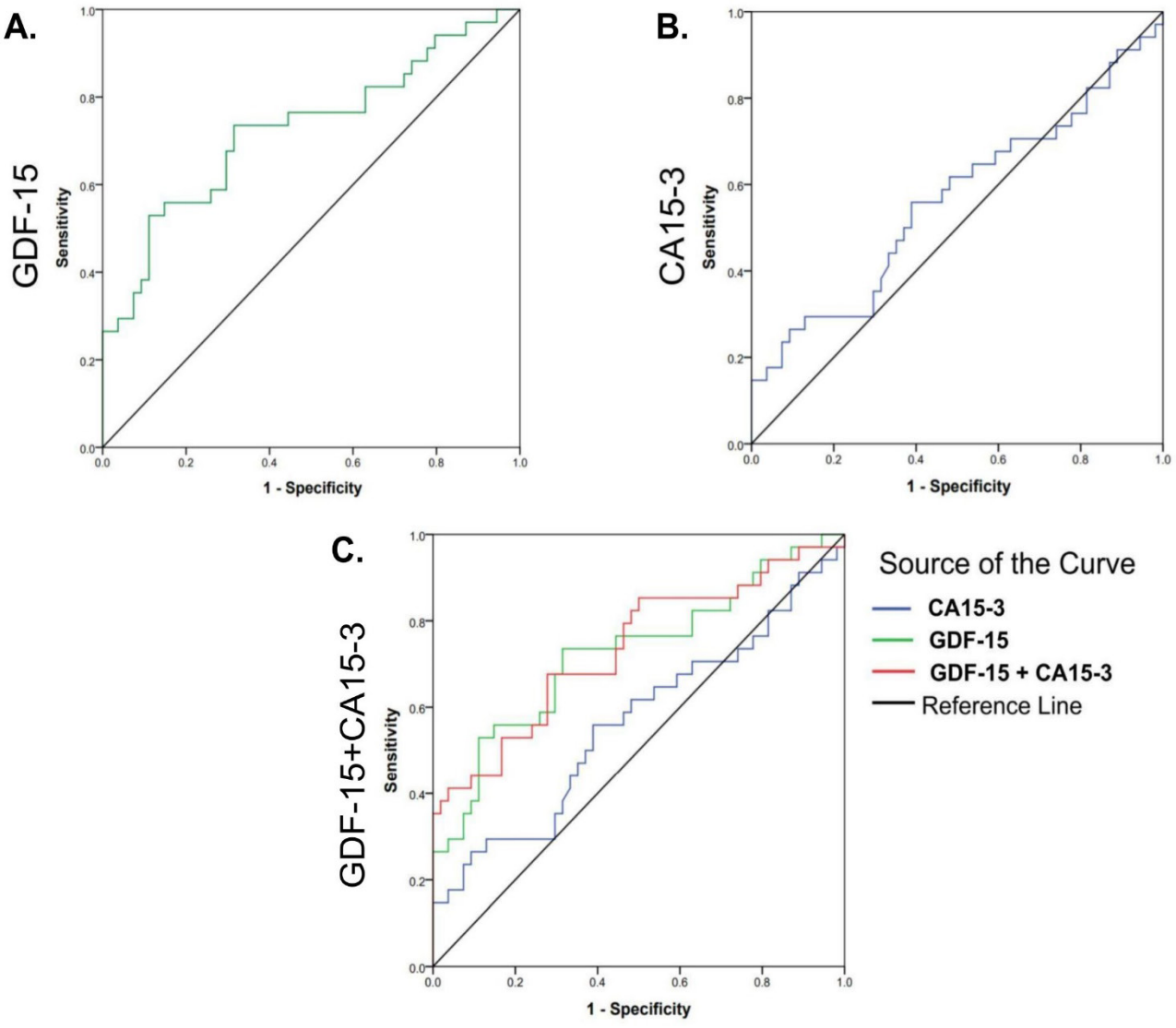
 # DeLong's test for comparison of two correlated Empirical ROC curves. While GDF-15 alone was not significantly better (p=0.389) than CA15-3 for breast cancer, the combined AUC of the two markers showed significant improvement over CA15-3 alone (p=0.003) but not GDF-15 (p=0.057). For early-stage breast cancer, the AUC of GDF-15 alone (p<0.001) and a combination of GDF-15 and CA15-3 (p<0.001) were comparable.

Table 4 Predictive ability of serum GDF-15 in breast cancer, adjusted for age

Predictor	Unadjusted			Adjusted		
	Coefficient	OR (95% CI)	*p*-value	Coefficient	OR (95% CI)	*p*-value
GDF-15	2.021	7.548 (3.474-16.400)	<0.001*	1.751	5.763 (1.978-16.793)	0.001*
Age	-	-	-	0.195	1.216 (1.136-1.301)	<0.001*

** Statistically significant at p<0.05*

Figure 4 **Receiver Operating Characteristic (ROC) curve analysis for A. Serum GDF-15, B. Serum CA15-3, and C. the combination of both markers in early-stage breast cancer.**



The combined ROC curve has been plotted from the combined probabilities derived from logistic regression of serum GDF-15 and serum CA15-3 in the study population. The reference line signifies an AUC of 0.50. Serum GDF-15 had a higher sensitivity (73.53%, p<0.001) compared to CA15-3 alone and the combination of GDF-15 and CA15-3.

DISCUSSION

BC is among the most prevalent cancers among women. Currently, mammography is the standard method of early-stage diagnosis of BC (20). However, existing diagnostic markers have low sensitivity; and the development of a highly sensitive and non-invasive approach for early BC diagnosis is needed to complement existing detection methods, which will consequently improve the outcome of the disease (20,21). In this study, we identified a novel early-stage diagnostic marker of BC with a higher sensitivity than CA 15-3, an already known marker of BC. Further, a combination of these two markers showed better diagnostic performance. To our knowledge, this is the first study reporting the clinical value of serum GDF-15 in the diagnosis of early-stage BC.

Earlier studies have reported significantly higher expression of GDF-15 in BC (17). But the role of serum GDF-15 in BC diagnosis has not been optimally explored. Windrichova et al. (14) assessed 130 patients with different cancers, including BC, and observed circulating GDF-15 to be increased in metastatic cancer patients, with 65% sensitivity and 90% specificity, with the cut-off value of 1480 pg/mL. Another study has reported that increased serum GDF-15 in BC patients is associated with metastasis (17). We found that serum GDF-15 could differentiate metastatic BC from non-metastatic cases with a sensitivity, specificity and AUC of 50.00%, 82.14% and 0.66, respectively. In-vitro and in-vivo studies have reported that an increase in GDF-15 levels is associated with proliferation, invasion, migration, drug resistance and stemness of BC (16-18). We also observed a significant difference in the serum levels of GDF-15 and CA15-3 in BC patients compared to the control group.

There are various diagnostic markers for screening of BC, such as CA27-29, CA15-3, carcinoembryonic antigen (CEA), which usually are not elevated in the control subjects but are found to be elevated in cancer patients. These markers are progressively increased in disease progression and recurrence and can monitor response to therapy (22). Among the known blood markers of BC, only CA15-3 is specific since it showed no elevation in patients in the control group (23). In other studies, elevated CA15-3 levels have been associated with tumour size, axillary node involvement, and advanced stages, making it a highly predictive prognostic marker (6,24,25).

Additionally, some studies report the role of CA15-3 in combination with other markers like CEA, CA125, Apurinic/apyrimidinic endonuclease (APE), epidermal growth factor receptor (EGFR), Neuron-specific enolase (NSE) for BC, but all these have less sensitivity and specificity (23,24,26). In our study, we also found the specificity of CA15-3 to be very high. But the main disadvantage of these markers is the lack of sensitivity for low volume disease. So, it is of no value in either screening or diagnosing early BC. Bayo et al. (23) have previously reported an AUC of 0.918 from a combined model, including CA15-3. The cut-off point was 0.697; this model had high sensitivity (85.7%) and specificity (82.3%).

In our study, serum GDF-15 in combination with CA15-3 had a better diagnostic value with sensitivity and specificity of 63.06% and 98.15%, respectively. We also observed that serum GDF-15 could also be used as an independent early diagnostic marker of BC and had a better predictive ability than CA15-3. Various other studies have reported that serum GDF-15 could be used as a diagnostic and prognostic marker of multiple types of malignancies such as liver, lung, prostate cancer (11-13,15). Previous studies showed that IHC positive GDF-15 tissue is correlated with high-grade tumours. GDF-15 expression was also positively associated with lymph node

metastasis (17). Reports have also demonstrated the association of GDF-15 with the advancement of BC. We observed that serum GDF-15 was significantly higher in distant metastatic patients than non-metastatic BC patients.

Welsh et al. (27) studied ten different tumour types and found GDF-15 to be increased in metastatic colorectal, prostatic, and BC compared to controls. Further, Wollmann et al. (28) found higher GDF-15 expression in breast tumour tissue samples compared to matched adjacent control tissues. Thus, even though having a small number of samples, these studies showed elevated GDF-15 expression in more than half serum or tissue specimens. Sasahara et al. (18) also reported higher GDF-15 expression in BC tissue than controls and higher GDF-15 expression in HER2-positive tumours. Finally, Peake et al. (17) found significant positive associations between GDF-15 expression and high tumour grade and ER-negative and HER2-positive status.

Our study takes these findings further and evaluates serum GDF-15 levels in circulation to differentiate BC from healthy controls. It can indeed be utilized in clinical settings as an adjunctive marker for these patients with better sensitivity and sensitivity.

There are some limitations of this study. Firstly, we could not follow up with the patients. Therefore, survival data analysis was not possible, which would have allowed us to present the prognostic role of GDF-15 in the BC patients. Secondly, IHC data of receptor status was missing for some patients. Thirdly, along with BC, some patients had comorbidities such as hypertension and diabetes mellitus, which could have affected the serum GDF-15 levels as GDF-15 is known to be increased in diabetes mellitus (29,30).

CONCLUSION

Biomarkers have long been prevalent in the clinical practice related to BC. However, early diagnosis can aid in better prognostic outcomes in these individuals. Serum GDF-15 has shown much promise as an early diagnostic marker in other cancers. Based on the existing evidence and our findings, we suggest that it can also be utilized as an adjunct marker in BC diagnosis, especially to detect early-stage (stage I-II) BC from the non-cancer group. The current study also suggested a cut-off (525.77 pg/ml) value differentiating BC and control groups. Future studies on a larger scale are needed to establish the robustness of serum GDF-15 use in a clinical setting.

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In-house algorithm for reporting discrepant HbA1c result and troubleshooting a case of false low HbA1c

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false low HbA1c, high performance liquid chromatography (HPLC), discrepant HbA1c, Bio-Rad D-10 HPLC

ABSTRACT

We report an unusual case of a patient having low glycosylated hemoglobin (HbA1c) below the reportable range, despite having borderline fasting blood glucose. The patient had decreased erythrocytes count and elevated reticulocyte count, with no evidence of hemoglobinopathy.

He reported taking multidrug therapy for borderline lepromatous leprosy. Dapsone induced hemolysis was identified as the cause for the discordant HbA1c. Thus, it is important to be aware of medications and conditions that may lead to a falsely low HbA1c level so that incorrect treatment decisions are not made. In such situations, alternative measure of glycemic control, such as fructosamine is recommended. Further

it is also recommended that clinical laboratories have standard protocol to troubleshoot any discrepant HbA1c result.

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INTRODUCTION

The application of hemoglobin A1C (HbA1c) result as a glycemic control indicator relies on glycation efficiency, which is determined by the integrity of globin chains of adult hemoglobin and life span of the erythrocytes. There are several clinical situations where globin chain or erythrocyte life span are affected and HbA1c is spuriously high or low and thus is not reflective of the true glycemic control. The most encountered situation in our laboratory is a low HbA1c result discrepant with blood glucose measurement. Our laboratory has established and implemented a reporting algorithm to approach any cases of discrepant HbA1c in relation to blood glucose measurement. (Figure 1)

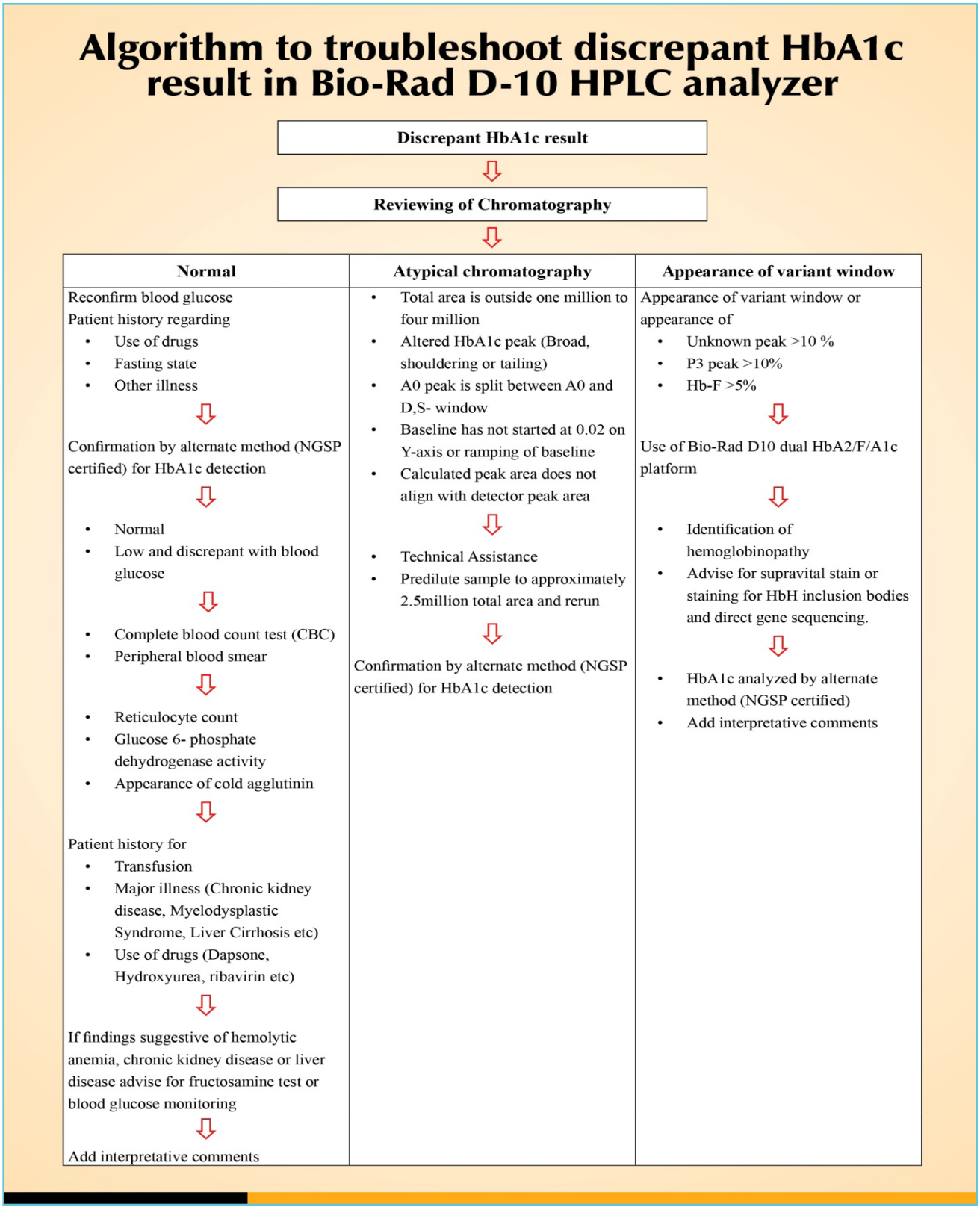
Our laboratory uses BioRad D-10 high performance liquid chromatography (HPLC) analyzer (*BioRad Laboratories Inc., Hercules, CA, USA*) with manufacturers provided retention time system for correct HbA1c and adult hemoglobin (HbAo) peaks identification. The reportable range for HbA1c is 3.7% - 18.4%. (17-178 mmol/mol). Variant hemoglobin and hemolytic disorders comprises the majority of cases with false HbA1c results.

Nepalese population harbors significant numbers of individuals with hemoglobinopathy, with the most common being sickle cell anemia and beta thalassemia. (1) In case of variant window detected in a chromatogram, the laboratory uses Bio-Rad D-10 HPLC system in short program mode (Variant II Beta Thalassemia Short Program, *BioRad Laboratories Inc., Hercules, CA, USA*) for the percent determination of hemoglobin A2, F and A1c and detection of any abnormal hemoglobin under the conditions specified by the manufacturer. HbA1c result is reportable if hemoglobin S trait and hemoglobin C trait is identified when sample is run in a Bio-Rad D-10 variant II beta thalassemia short program mode. (2) Similarly, HbA1c result is reportable if hemoglobin E trait and D trait is detected, unless a degradation peak (Unknown peak) is identified between HbA1c and P3 peaks in a chromatogram. (2) P3 denotes the level of degraded hemoglobin and should be less than 10% for the HbA1c result to be reported. In conditions with S and C window in a chromatogram, HbA1c is not reportable. (2) In such cases, HbA1c is analyzed by alternate method using boronate affinity chromatography (NycoCard™ HbA1c test, *Abbott Diagnostic technologies AS, Oslo, Norway*) or nephelometry (MISPA i3, *Agappe Diagnostic Switzerland*). These are National Glycohemoglobin Standardization Program (NGSP) certified methods traceable to the Diabetes Control and Complications Trial (DCCT) reference method used as back up at our laboratory. (3)

Complete blood count (CBC) testing is done in cases where technical error or variant window is not detected but HbA1c is spuriously low or high, to rule out anemia as a potential cause of discrepant HbA1c result. Serum iron chemistry and testing for vitamin B12 is advised if CBC findings are suggestive. Based on the CBC findings, peripheral smear (PS) examination and reticulocyte count is done in selected cases to rule out possibility of hemolytic disorder. If features suggestive of hemolysis are seen then, Glucose-6-phosphate dehydrogenase (G6PD) activity is determined by CareStart TM G6PD Biosensor Analyzer (*Access Bio Korea, Inc*) at our laboratory. The prevalence of G6PD deficiency in Nepal is 3.5% and this percentage varies according to the ethnicity. (4)

Apart from this, patient history is taken for any recent blood transfusion; presence of any

Figure 1 Algorithm to troubleshoot discrepant HbA1c result



hematological condition or other major illness and use of any drugs having potential to interfere with HbA1c report. Finally, the respective HbA1c result is reported with comment and additional laboratory findings, to facilitate physician's proper HbA1c result interpretation. The majority of cases that need troubleshooting consist of variant hemoglobin and hemolytic anemia where testing HbA1c by alternate method and monitoring glycemic status by alternate method such as fructosamine is advised respectively.

This report highlights the limitations of using HbA1c as a diagnostic tool in a patient using Dapsone for leprosy treatment. Furthermore, this report emphasizes the careful interpretation of each measurement of HbA1c in context of a clinical situation.

CLINICAL DIAGNOSTIC CASE

We received a blood sample of a 28 years male for investigating HbA1c level. The patient's result was 3.4% (13.66 mmol/mol) which was below the reportable range. His fasting blood glucose was 5.6 mmol/L (3.89 – 5.55 mmol/L). Since his HbA1c result was discordant with the fasting blood glucose and was not in the reportable range, the possibility for presence of hemoglobinopathy was suspected. We used Bio-Rad D-10 HPLC system (Variant II Beta Thalassemia Short Program, *BioRad Laboratories Inc., Hercules, CA, USA*) for the percent determination of hemoglobin A2, F and A1c and detection of any abnormal hemoglobin under the conditions specified by the manufacturer. The chromatogram was normal. His blood sample was also investigated by alternative methods for HbA1c analysis. The result was still 3.1% (10.38 mmol/mol) and 3.3% (12.57 mmol/mol) by nephelometry (MISPA i3, *Agappe Diagnostic Switzerland*) and boronate affinity chromatography method (NycoCard™ HbA1c test, *Abbott Diagnostics Technologies AS, Oslo, Norway*).

Since his HbA1c was very low and was discordant with the plasma blood glucose in three different assay platforms, the cause for false low HbA1c was further investigated. The CBC test and examination of PS was done as per our established protocol. Patient had normal hemoglobin but reduced red blood cells count and increased mean corpuscular volume (MCV) (Table 1). Anisopoikilocytosis and normochromia along with occasional microspherocytes were observed in the peripheral smear test. Patient had high reticulocyte count.

Since he had a hemolytic picture upon hematological investigation, the biochemical markers for hemolysis were done. High lactate dehydrogenase, high total and indirect bilirubin and reduced haptoglobin further suggested the presence of hemolysis. (Table 1) His renal function test, Glucose-6-phosphate dehydrogenase (GPPD) activity and thyroid function test was normal. Considering the possibility of hemolysis, detail patient history was taken.

The patient was a known case of Borderline lepromatous Leprosy. He was taking Gabapentin 300 milligram, Cyanocobalamin 1500 microgram and Prednisolone 10 mg daily for peripheral ulnar neuritis along with medications for leprosy (Rifampicin 600 mg monthly, Dapsone 100 mg daily and Moxifloxacin 400 mg daily). There was no family history of any hematological conditions.

On reviewing the literature, we found various reports on dapsone leading to a falsely low HbA1c. The drug dapsone has been listed in a troubleshooting algorithm, due to a significant number of cases reported from our part of world. (5, 6)

Fructosamine level of the patient was assessed, and it was found to be high (Table 1). Interpretative comments about unreliability of

Table 1 Laboratory investigations

Parameters	Result	Reference range /Unit
Hemoglobin	144	135 - 169 g/L
Red blood cells (RBC) count	4300	4400 - 5600 G/L
Mean Corpuscular Volume (MCV)	106.3	81.8 - 95.5 fL
Mean Corpuscular Hemoglobin (MCH)	33.2	27 - 32.3 pg
MCV Concentration (MCHC)	31.5	32.4 – 35 g/dL
Platelet count	100	150 – 450 G/L
Reticulocyte count	4.8	Up to 2 %
Packed cell volume (PCV)	45.7	42 – 52 %
Erythrocyte Sedimentation Rate (ESR)	06	0 – 22 mm/hr
Total Bilirubin	39	5 –31 µmol/L
Direct bilirubin	5	0 – 5 µmol/L
Aspartate Transaminase (AST)	25	17 – 59 U/L
Alanine Transaminase (ALT)	32	10 – 45 U/L
Alkaline Phosphatase (ALP)	348	98 – 279 U/L
Lactate Dehydrogenase (LDH)	345	100 – 210 U/L
Haptoglobin	0.28	0.45–2.05 g/L
Fasting Blood Glucose (FBG)	5.6	3.89 – 5.55 mmol/L
Fructosamine	317.40	205 – 285 µmol/L
Cortisol (8 am)	22.62	137.95 – 634.57 nmol/L
Adrenocorticotropin Hormone (ACTH)	4.18	< 10.13 pmol/L

Vitamin B12	812.54	138.01 – 614.75 pmol/L
Vitamin D	56.41	74.88 – 249.6 nmol/L
Iron	17.3	13 – 31 µmol/L
Ferritin	270.2	21.81 – 274.66 µg/L

HbA1c for monitoring glycemic status in the index patient and advice on testing for plasma glucose or serum fructosamine level was added to the report. Informed consent has been taken from the patient for the publication of this case report.

DISCUSSION

Shortened erythrocytes life span and rapid red cell turnover as seen in patients using dapsone, might have low HbA1c level but not indicating good glycemic condition due to inefficient and lowered degree of glycation. There are several case reports of falsely low HbA1c levels in patients on dapsone, including reports of patients infected with the human immunodeficiency virus (7), patients with polychondritis (8), organ transplant patients (9) patients diagnosed with leprosy (10) and patients using dapsone for prophylaxis against *Pneumocystis jiroveci* pneumonia (11).

Dapsone, also known as diaminodiphenyl sulfone, is an antibiotic commonly used in combination with rifampicin and clofazimine for the treatment of leprosy. It is a second-line medication for the treatment and prevention of pneumocystis pneumonia and for the prevention of toxoplasmosis in those who have poor immune function. Additionally, it has been used for acne, dermatitis herpetiformis, and various other skin conditions. Dapsone leads to a false low HbA1c via various mechanisms such as by inducing hemolysis, promoting the oxidation of hemoglobin to methemoglobin which interferes with the HPLC assay and finally by reducing erythrocyte survival independent of its hemolytic effect (12,13,14). The index patient had elevated reticulocyte counts, decreased haptoglobin, elevated indirect bilirubin and elevated lactate dehydrogenase levels, which are indicative of hemolysis. He also had anisopoikilocytes and microspherocytes noted in the PS examination reflecting hemolytic disease. Hemolysis does not always result in anemia (15), as seen in our patient who had normal hemoglobin in spite of having decreased erythrocyte count. The high MCV in this patient is attributed to the high reticulocyte count since these cells are larger than mature erythrocytes. High level of vitamin B12 and low cortisol in the index patient is attributed to the chronic use of vitamin B12 and steroid.

Methemoglobinemia results in functional anemia where hemoglobin appears normal but the ability of hemoglobin to carry oxygen to the tissues is impaired thus resulting in severe symptoms such as headache, shortness of breath, fatigue, seizures and coma. Test for methemoglobin was not done for our patient; however, he was warned about the symptoms of severe methemoglobinemia. Thus, hemolysis and reduction of erythrocyte lifespan was a cause of misleadingly low HbA1c in the index case.

Since dapsone leads to a falsely low HbA1c, another measure of glycemic control is necessary in patients taking this medication. Fructosamine refers to proteins that are non-enzymatically

glycated via ketoamine linkages at the N-amino terminal. Since, albumin is a major plasma protein, fructosamine primarily reflects glycated albumin. Further, because the half-life of albumin is 2-3 weeks, fructosamine indicates recent glycemic status. The index patient had higher fructosamine level (Table 1). We present case of a patient who had very significant and spurious reduction in his HbA1c result occurring secondary to treatment with dapsone. Prompt recognition of the dapsone effect avoided inappropriate intervention in this patient.

LEARNING POINTS

HbA1c result that lies below the reportable range and is discrepant with the blood glucose result should alert laboratory physician about the conditions associated with shortened red blood cell survival which may result in an inaccurate HbA1c. Alternate methods to assess glycemic control, such as fructosamine or glucose monitoring, should be used in such cases. Clinical laboratory should prepare and follow the algorithm suitable to its assay design, to troubleshoot any discrepant HbA1c result. The addition of the interpretative comments when applicable for the ease of clinicians and patient safety is recommended.

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Conflict of interest

The authors declare that there is no conflict of interest in the publication of this manuscript.

Consent

Informed consent was taken from patient for the publication of this case report.

Authors' contribution

VP - Conceptualization and writing of the report

AS, DP, SP, KG - Scientific Content for the report

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A puffy child – a rare case of steroid resistant nephrotic syndrome with ANLN mutation

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CASE REPORT

Recent advances in genomics have uncovered the molecular mechanisms involved in the broad spectrum of variation associated with steroid-resistant nephrotic syndrome. Over 50 monogenic causes of steroid-resistant nephrotic syndrome have been discovered; however, these genes are implicated in only a small proportion of cases. Using a combination of whole-exome sequencing and genome-wide linkage studies, a missense mutation in anillin (ANLN) has been identified as a cause of focal segmental glomerulosclerosis, a pattern of glomerular injury associated with steroid-resistant nephrotic syndrome. We report a case of 2-year-6-month-old male child, who presented with severe edema and oliguria for 6 weeks. He was found to be an early steroid non-responder,

hence renal biopsy and genetic testing were ordered. These findings were in favour of focal segmental glomerulosclerosis, a common cause of childhood steroid-resistant nephrotic syndrome. It is important to identify the causative agent to avoid unnecessary immunosuppressive therapy and its associated risks.

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INTRODUCTION

Nephrotic syndrome (NS) encompasses a heterogeneous group of disorders characterised by massive proteinuria, hypoalbuminemia and edema. The most common glomerular disease of childhood is NS, with an incidence of approximately 1–2 per 100,000 [1]. Nearly 85% of pediatric NS cases respond to steroids, with the remaining 15% being steroid-resistant [2]. Steroid-resistant nephrotic syndrome (SRNS) may be characterised further based on renal histopathology, with almost 20% showing focal segmental glomerulosclerosis (FSGS) [3]. Steroid resistance and persistent proteinuria are key determinants of impending risk for end-stage renal disease (ESRD) [4].

According to recent evidence, genetic etiology is found in nearly 30% of SRNS cases [1]. Anillin Actin Binding Protein (ANLN) is one of the monogenic mutations responsible for SRNS. Anillin plays a pivotal role in cellularisation and cytokinesis. Mutation in ANLN causes upregulation of PI3K/AKT/mTOR/p70S6K/Rac1 pathway, elucidating its importance in pathogenesis of podocyte dysfunction in FSGS. ANLN has also been recognized as a driver of cellular proliferation in various forms of human tumours [5]. Therefore, it is crucial to expand our understanding of the pathobiology involved in the disease to design personalised treatment strategies.

CLINICAL CASE DESCRIPTION

A 2-year-6-month-old male child, born to non-consanguineous parents, presented with persistent facial swelling, abdominal distention and reduced urine output for 6 weeks. He had recent onset breathing difficulty. He was a known case of NS being treated with prednisolone 2 mg/kg/day. His antenatal and postnatal history were uneventful. His nutrition and immunization status were appropriate for his age. He had nil significant family history.

On examination, the child was active and alert. Anthropometric examination recorded findings appropriate for his age. Examination of vitals revealed tachycardia (heart rate: 108/min), tachypnoea (respiratory rate: 27/min) and oxygen saturation of 93% in room air. He had elevated blood pressure of 130/90 mmHg (>95th percentile for his age and height) and pitting pedal edema.

Routine blood investigations, including renal and liver function tests were within normal limits. His baseline coagulation study and thyroid function test were normal. Urine analysis revealed nephrotic range proteinuria (4+ on dipstick and elevated protein creatinine ratio of 15.78) and microscopic hematuria. He also had hypoalbuminemia (1.4 g/dL) and hypercholesterolemia (1427 mg/dL). Ultrasound showed moderate pleural effusion, ascites and enlarged hyperechogenic kidneys. Suspecting SRNS, renal biopsy was performed, which revealed features of FSGS (Figure 1). In view of severe anasarca and respiratory distress, he was initiated on albumin infusion with 20% albumin, 1 g/kg followed by diuretics, furosemide 1mg/kg/dose. After 2 sessions of combined albumin and diuretic therapy, edema settled and albumin level increased to 2 g/dL. Genetic study using targeted exome sequencing, revealed heterozygous missense mutation in exon 14 of ANLN gene that results in amino acid substitution of

methionine for threonine at codon 821 (p.Thr 821Met;ENST00000265748.7). Currently, he is being treated with tacrolimus 0.1 mg/kg/day and enalapril 0.3 mg/kg/day, along with normal protein and no added salt diet. His parents have been counselled about the disease course, trial of immunosuppressive therapy and the need for renal transplantation in future.

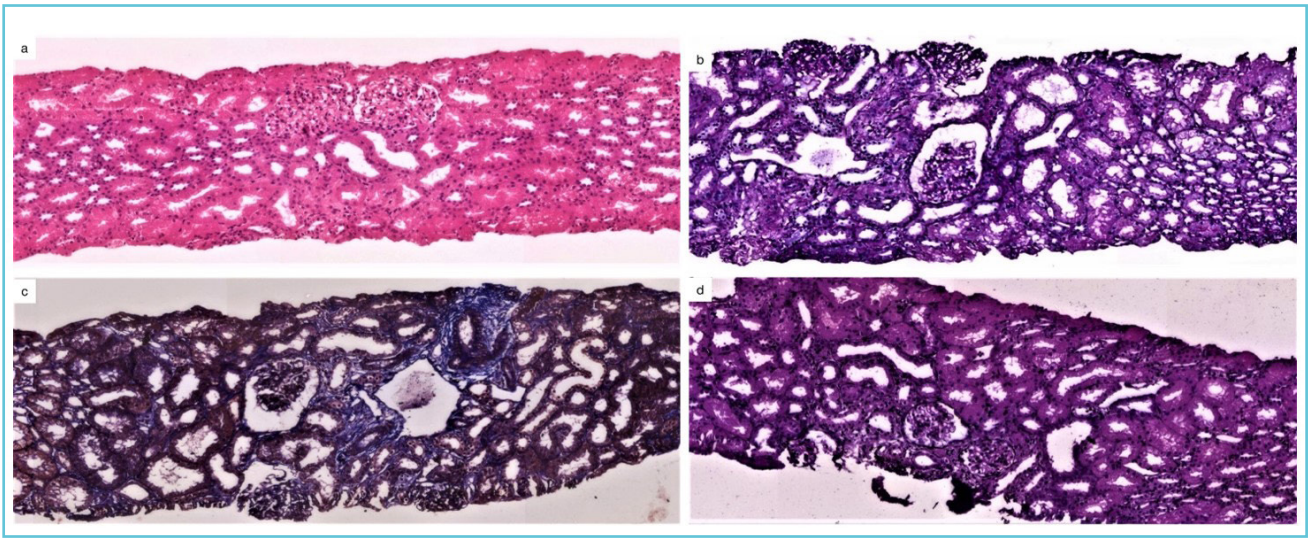
DISCUSSION

Nephrotic syndrome (NS) is a non-inflammatory disorder of glomerulus, characterised by increased glomerular leakage of proteins, predominantly albumin. It is denoted by a group of features including edema, proteinuria and hypoalbuminemia. Corticosteroids are the mainstay of therapy for NS; however, approximately 15% of patients fail to attain remission even after 4 to 6 weeks of daily prednisolone and are classified as steroid-resistant nephrotic syndrome [2].

Glomerular filtration barrier (GFB) is a multifaceted apparatus composed of specialized fenestrated endothelium, podocyte, slit diaphragm, and glomerular basement membrane (GBM). Podocytes are highly specialized epithelial cells, which play a vital role in structural and functional integrity of GFB. These podocytes, along with their interdigitating foot processes are connected together with the aid of slit diaphragm. This ultrastructure of GFB regulates the ultrafiltration of molecules, thereby preventing excretion of albumin and other large plasma proteins. Dysfunction of any component of the GFB, can result in severe proteinuria leading to NS [1,6].

Mutations in genes encoding podocyte-associated structural proteins have been demonstrated in approximately 30% of childhood SRNS. ANLN, a multi-domain protein, found to

Figure 1 **Histopathological findings of renal biopsy showing features of focal segmental glomerulosclerosis**



a) Two Glomeruli showing mesangial hypercellularity and segmental capillary lumen obliteration and sclerosis (H&E; x 400).

b) Periodic Acid Schiff stain highlighting the sclerosed glomeruli and focal tubular atrophy (PAS x 400).

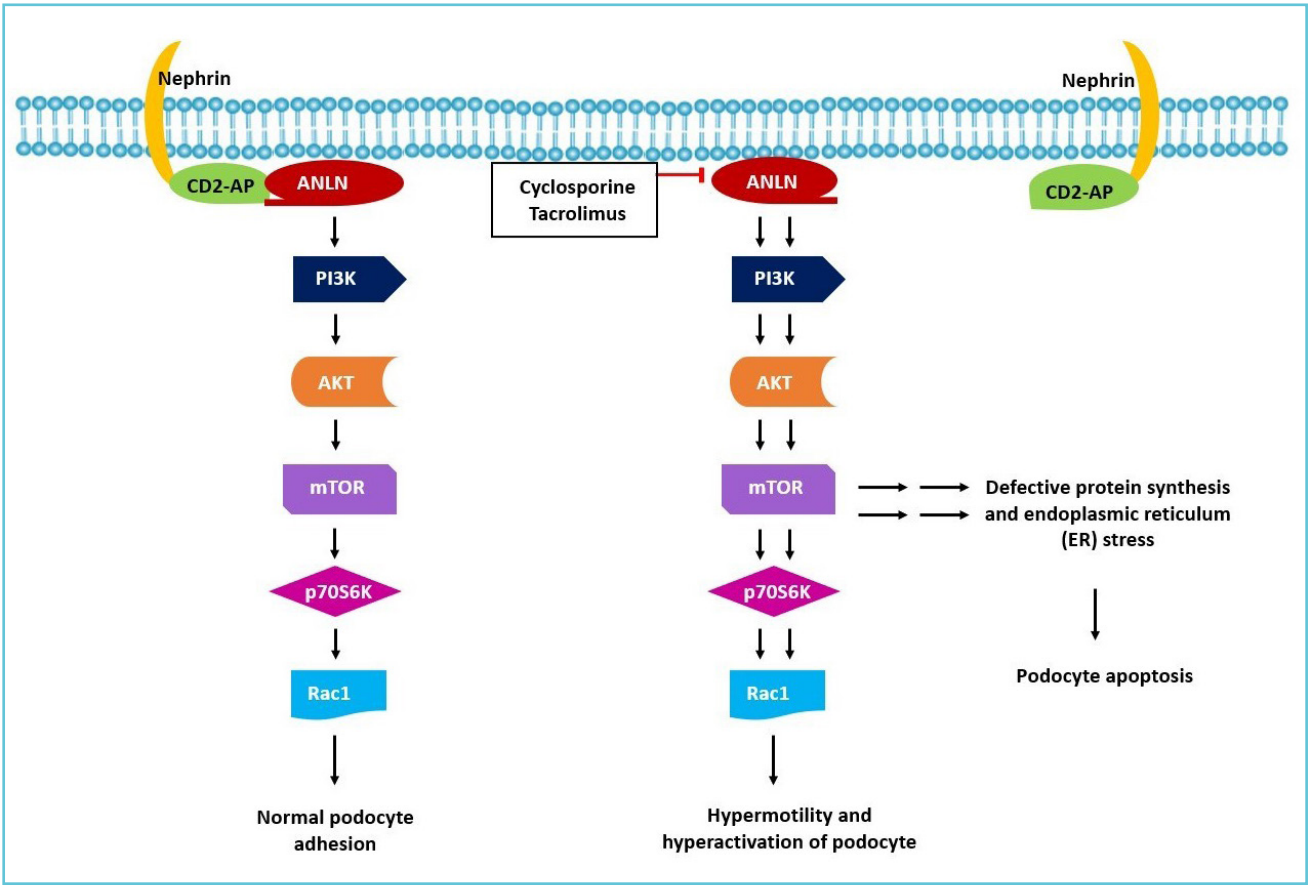
c) Masson Trichrome stain showing mesangial hypercellularity, tubular cyst formation and medial wall hypertrophy of the blood vessels confirming the vascular changes secondary to hypertension (MT x 400).

d) Jones Methenamine Silver also highlights the segmental glomerular sclerosis (JMS x 400).

have autosomal dominant inheritance is typically associated with adult-onset FSGS [1]. The presenting child has early-onset SRNS associated with monogenic mutation in ANLN gene. In silico analysis, predictions of ANLN variant were found to be damaging by SIFT (Sorting Intolerant From Tolerant), Polyphen-2 (Polymorphism Phenotyping V-2) and LRT (Likelihood Ratio Test).

Anillin, coded by ANLN gene is one of the podocyte-associated protein responsible for maintaining the integrity of podocyte actin cytoskeleton. Studies performed in vitro using immortalized human podocytes and in animal models suggest that loss-of-function mutation in ANLN disrupts podocyte cytoskeletal dynamics and promotes podocyte apoptosis through hyperactivation of PI3K/AKT/mTOR/p70S6K/Rac1 pathway. In contrast with intact anillin, the mutant displays a significantly reduced binding affinity to the slit diaphragm-related protein, CD2-associated protein (Figure 2). These findings form the basis for pathogenesis of FSGS, a clinicopathological entity characterized by NS,

Figure 2 **Flowchart depicting the activation of PI3K/AKT/mTOR/p70S6K/Rac1 pathway by ANLN**



Flowchart depicting:

(i) Normal activation of PI3K/AKT/mTOR/p70S6K/Rac1 pathway by intact ANLN (Left)

(ii) Hyperactivation of PI3K/AKT/mTOR/p70S6K/Rac1 pathway by mutant ANLN resulting in defective protein synthesis and podocyte apoptosis, causing FSGS (Right), and

(iii) Site of action of calcineurin inhibitors (cyclosporine and tacrolimus).

with segmental sclerosis of glomeruli and effacement of podocyte foot processes. In addition, ANLN is upregulated in diverse site-specific human tumours including brain, lung, breast, renal, ovarian, endometrial, liver, pancreas, colorectal and bone marrow cancers [7].

A study published in Journal of American Society of Nephrology, involving 250 families with FSGS revealed ANLN mutation in a family of 26 members. After performing whole-genome sequencing, 9 members were found to have ANLN mutation. One of the family members had childhood-onset FSGS, five members progressed to ESRD and received renal transplantation but none of them had post-transplant disease recurrence [7].

The hallmark of NS is massive urinary loss of proteins, especially albumin. Other features include generalized edema, hyperlipidemia and hypoalbuminemia. Less frequent symptoms include hypertension, hematuria and oliguria. In addition, children with NS are prone to infections, acute kidney injury and thromboembolic events [4].

The diagnostic criteria of NS include massive proteinuria (3+ or 4+ in dipstick or >40 mg/m^2/hour), hypoalbuminemia (albumin <3 g/dL) and generalised edema. Renal biopsy has authentically been utilised as a main diagnostic and prognostic indicator for children with SRNS. In cases of early-onset SRNS, genetic testing has obviated the need for renal biopsy and serves as a less invasive diagnostic modality. Identification of mutation is important for genetic counselling and possible antenatal screening for future pregnancies, prediction of prognosis and post-transplant disease recurrence, and surveillance of other extra-renal phenotypes [2,8]. In addition, further discovery of novel genes will improve our understanding about the pathogenesis of SRNS and allow for a pragmatic approach to therapy.

Steroid-resistant nephrotic syndrome is a highly heterogeneous disease, with over 60 known disease-causing genes. Using gene panel analysis, all protein-coding exons of multiple genes can be tested simultaneously using high-throughput polymerase chain reaction amplification and sequencing techniques. While, whole exome sequencing allows screening of all protein-coding regions of genes in a genome, targeted next generation sequencing uses predesigned gene panels containing important genes or gene regions associated with a disease or phenotype such as SRNS, thereby decreasing costs and increasing efficiency. Even though high-throughput sequencing technologies have revolutionized the identification of mutations responsible for a diverse set of genetic disorders, the identification of causal mutations continue to remain ambiguous in a significant proportion of patients. This could be partially due to pathogenic variants being located in non-coding regions (introns), which are largely missed by targeted exome sequencing. With the advent of whole-genome sequencing, detection of non-coding variations has become possible [7,9]. In our patient, extended gene panel analysis suggested ANLN mutation as the underlying cause for SRNS with no other possible disease-causing mutations being identified.

The fundamental aim of treatment in children with SRNS is to improve the prognosis, thereby ameliorating progression to ESRD. Angiotensin converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARBs) can be employed to reduce proteinuria and lower blood pressure. The anti-proteinuric effect of ACEI/ARBs is predominantly due to post-glomerular vasodilation and pre-glomerular vasoconstriction, resulting in reduced filtration pressure, thereby lowering the leakage of proteins through GBM [10]. Statins can be used for management of hyperlipidemia in children with NS. The presenting child is being managed with

dietary modifications, since the use of statins is not warranted in children less than 8 years of age [11]. Parents have been counselled about lipid apheresis as an alternative treatment option, if lipid levels do not normalise with dietary modification and other supportive measures.

In children with SRNS, the treating nephrologist may choose to use non-glucocorticoid agents to induce complete or partial remission. There have been in vitro studies proving the potential benefits of calcineurin inhibitors in treatment of FSGS with ANLN mutation (Figure 2) [5]. The presenting child has been started on tacrolimus and will be continuously monitored until remission. The therapy will be stopped if he does not attain partial or complete remission within 6 months.

Albumin infusion along with diuretics, is considered as a therapy in cases of refractory edema [12,13]. The rationale behind albumin infusion relies upon "underfill" theory, i.e., hypoalbuminemia (reduced oncotic pressure) decreases intravascular-to-interstitial albumin gradient creating a surge in the movement of fluid from intravascular compartment into interstitial space. The infused albumin normalises the oncotic pressure and as a result, edema fluid is drawn-back into circulation, from where it is excreted by the kidneys with the help of diuretics.

According to PodoNet registry, 3 out of 4 children with monogenic SRNS progress to ESRD, requiring dialysis or renal transplantation. Fortunately, several studies have accentuated a negligible risk of post-transplant disease recurrence in patients with genetic forms of SRNS, compared to a whopping 30% in children with non-genetic forms of SRNS [14].

With ever-increasing number of genes involved in SRNS, the need for understanding the molecular mechanisms and genotype-phenotype correlations is inevitable. Given the heterogeneity of the disease, taking up next generation sequencing-based "bench to bedside" translational approaches would bring about revolutionary outcomes in its diagnosis and management.

LEARNING POINTS

1. Genetic testing should be performed in all children non-responsive to prednisolone therapy to help the treating clinician to provide personalised treatment options, possibly avoiding unnecessary immunosuppressive therapy.
2. Parents of children with SRNS should be counselled about the disease course and need for renal transplantation in future.
3. Children having monogenic forms of SRNS may be predisposed to certain other syndromes or site-specific tumours. Therefore, the treating pediatrician should be vigilant in monitoring these children for early disease detection.

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Ethical approval

All procedures were performed in accordance with the ethical standards of the institutional research committee at which the study was conducted and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Hope injections: the promises of regenerative medicine in curing type 1 diabetes mellitus

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LETTER TO THE EDITOR

One evening of 2006, my mother announced that scientists had made a groundbreaking discovery. By 2012, we would be having the first “vaccine” against type 1 diabetes mellitus (T1D), and so my chronic disease would finally come to an end. It was the most fascinating news I had heard as a 9-year-old.

T1D is a chronic autoimmune endocrine condition, caused by a faulty recognition of self and foreign antigens by the immune system[1]. It attacks the insulin-producing beta cells of the islets of Langerhans in the pancreas; insulin is a vital hormone for blood glucose control. Without it, patients must turn to insulin injections multiple times daily, adjusting doses to fluctuations in food, activity and numerous other

interdependent factors. Blood sugar monitoring is also imperative. But oftentimes, exogenous insulin will not act as deftly as the pancreas, causing the unpleasant symptoms and dangerous consequences of hypoglycaemia: fatigue, dizziness, trembling and even seizures and coma[2].

The truth is, life with T1D requires constant adaptation and planning ahead. I may not have known at the time, but walking out of the hospital with my diagnosis, I had acquired a monkey on my back, for life. Today, nearing the end of my medical studies, I realize that T1D management resembles applying evidence-based medicine 24/7. Contrarily to common belief, experience does not always automate management, while type 1 diabetic patients lead a life with the astonishing number of up to 180 extra health-related decisions per day[3]. Nutritional training and constant alertness are vital, day in, day out.

2012 came and went. Today, more than 1.25 million Americans are affected[4], a reality that seems odd, considering the massive accomplishments in Regenerative Medicine. Upon the recent outbreak of the COVID-19 pandemic, individuals with T1D have been reported to be at higher risk for severe illness[5] and in-hospital death[6] due to COVID-19. On top of that, the endocrine tropism of SARS-CoV2, the virus causing COVID-19, offers a potential explanation for the observed link between the infection and increased incidence of T1D[7]. Beyond health complications, for many low or middle-income countries (LMICs), and even for patients in high income countries with private healthcare systems, T1D constitutes a heavy financial burden, with essential insulin and technology often being unaffordable[8]. More often than not, physicians are faced with diagnostic challenges regarding the pathophysiological mechanism of diabetes mellitus. For example, some patients are erroneously diagnosed with T1D and treated with insulin, while having a different, rare form of monogenic diabetes: Maturity onset diabetes of the young (MODY), which often manifests with comparable clinical characteristics to T1D, but without an autoimmune origin. Genetic testing is required and treatment usually involves diet, sulfonylureas or metformin[9]. Evidently, MODY should be included in the differential diagnosis of every case of atypical manifestation of T1D.

Even though my medical background has now enabled me to grasp why unimagined roadblocks would not have allowed for a cure of T1D in 2012, the cumulative global progress seems poised to ultimately take the disease to meet smallpox in history books. Planning to specialize in diabetes research and management, I wish to contribute to finding a cure for the disease that has been my life's greatest challenge.

The leading strategy today is beta cell replacement, though only therapeutically available for a small fraction of patients [10-12]. It can be achieved by transplanting self or allogeneic stem cells, differentiated into specialized insulin-producers. Replenishing pancreatic islets will theoretically reverse the deficiency, but certainly, that is easier said than done. Difficulties appear from *in vitro* stages. The engineered beta cells often exhibit immature metabolism and insulin kinetics which deviate from the normal glucose-dependent secretion pattern. A key point, yet to be clarified, is whether the artificial islets should include beta cells only, or other endocrine islet cells, too. Embracing the idea that all these types of cells were phylogenetically preserved in adjacent sites advantageously, most protocols include integral islet-like clusters, but the optimal ratio remains an open question[13].

However, even if we inject the best, functional islets, there are further setbacks ahead. One risk is the development of malignancy, if incompletely differentiated cells are accidentally co-transplanted[14]. Moreover, transplants can be

immunogenic, meaning that, the immune system will lurk on two sides. The new cells might be *different enough* from the host's to provoke an allogeneic rejection response, or *similar enough* to trigger the autoimmunity roller-coaster again[15]. So far, the only option has been immunosuppression, with the well-known severe risk of susceptibility to infections and malignancy[16].

An elegant alternative is wrapping transplants in immune-proof devices (macro-encapsulation) or hydrogel-based biomaterials serving as molecular coats (micro-encapsulation) [17-19]. Yet, it remains a bioengineering challenge, as the desirable composition must be impermeable to attack, but permissive to the secretion of insulin and the exchange of oxygen and nutrients. Over time, capsule architecture has been debated. Previously, microcapsules were larger than islets, allowing poor contact. Improved designs have now hit the labs, able to wrap around islets and conform to their shape and size[19,20].

Even without coats, transplants can be processed with the help of gene-editing tools such as CRISPR-Cas9 to "ninja cells", which are devoid of surface proteins and evade immune recognition and attack[21]. These might have another advantage: They could constitute universal cell donors, with minimal immunogenicity, taking us closer to industrial islet production. However, escaping immune surveillance must not be taken too far: If cell division aberrations occur, the immune system would not be able to prevent malignancy stemming from the transplants. Subsequently, scientists are attempting to add a suicide protein, activated upon administration of a certain drug, to serve as a safety valve[22].

Moving from disguise to adaptation, immunomodulation trials have been in place[23], and may reduce the need for immunosuppression more promptly than encapsulation. Such an agent, Teplizumab, has reached final stages in multinational clinical trials[24], both to reverse overt T1D, and to prevent clinical manifestation in individuals at-risk of developing it, meaning siblings of T1D patients with a considerable titer of islet-specific autoantibodies but so far preserved islet function[25].

But where to plant our little insulin factory? So far, experiments have involved the omentum (a large membrane covering the intestines), subcutaneous tissue and the portal vein, as locations differ in their ability to generate vasculature[22]. Especially the portal vein, although a convenient choice for transplantation, entails exposure of the islets in maximum concentrations of nutrients and drugs, as per human physiology, and that can be harmful to cells used to surviving protected in the pancreas. Another interesting approach involves the interaction of islets with the host microbiome[26]. Among other institutions, the Joslin Diabetes Center is testing this, after their striking Medalist Study revealed a percentage of patients, who were somehow protected against complications, after 50 years with the disease[27]. This leads to the hypothesis that Precision Medicine may have an important role in creating effective cures. As exciting as it may sound, it introduces a new level of challenge, with growing evidence that T1D immunopathology varies among patients[9]. Table 1 shows a summary of the main therapeutic targets and approaches to restore or preserve beta cell function in T1D currently under laboratory development or in clinical trials, discussed in this article.

Nevertheless, while we wait for definitive therapies, we luckily have the technologies of continuous glucose monitoring (CGM) and insulin pumps making life with T1D easier[28]. Still, as exquisite as they are, they entail a heap of information for the patient. The dual-hormone iLet Bionic Pancreas pump seems like an "external electronic version" of our previously described mixed-cell islet: Along with insulin, the pump delivers glucagon - the insulin counteracting hormone, micro-adjusting their balance every

few minutes, just like a real pancreas. Its goal is to automate glucose control and it may soon enter the market, now that the stability of glucagon in room temperature is finally optimized[29].

Overcoming all these diverse obstacles may be frustrating, but the concepts we are now handling seemed like science fiction twenty years ago. I speculate that the cure, once perfected, will seem to future generations like Oedipus' solution to the riddle of the Sphinx: a solution so sensical, which however, only he was able to conceptualize. A prominent scientist devoted his career to T1D research after his two children were inflicted[30]. My life motto will be his answer when asked whether he thinks we will find a cure: "I am not going to give up until we do". In fact, I already feel as part of the efforts of the T1D scientific community. When reading original research and hitting the key message, a little internal voice shouts "Eureka!", as if it were my own discovery to celebrate. These are hope injections, and thankfully they don't hurt.

Eventually, my disease came with an appreciation that healthy individuals own a miraculous pancreas. Still, while life-threatening[31], T1D is manageable. Interning in hospitals, I often see fatally ill patients, inflicted with ailments that can be destroyers. But me? I have the precious chance to fight. My gift was the inexhaustible desire for a cure, combined with the physical and mental ability to search for one.

My fervent hope is that someday, I will be privileged to know life without diabetes[32]. It is also that my patients will, too. I view my challenge as to continuously evolve *from a chronic worrier to a chronic warrior*, as one of my mentors put it. Can a system with such complexity be modified to work harmoniously, with no component falling short? The tools are all on our hands, and we are only facing an "assembly" puzzle. Personally, I can see myself tightening some screws, as we make this dream reality.

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Table 1 **Main therapeutic targets and approaches to restore or preserve beta cell function in T1D currently under laboratory development or in clinical trials**

Target	Approach
Islet cell replacement	Transplantation of embryonic, mesenchymal or induced pluripotent stem cells in various stages of differentiation into pancreatic islets
Protection from immune destruction	Micro- or macro- encapsulation
Development of minimally immunogenic transplants	Transplant cell engineering through gene editing
Mitigation of autoimmunity in T1D confirmed patients Prevention of clinical disease for at-risk individuals	Selective immunosuppression – immunomodulation (eg, Teplizumab)

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