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Editorial

Technology, Molecular Diagnostics and Current Challenges in Educating Laboratory Professionals and Clinical Practice

Patricia Tille Ph.D MLS(ASCP) AHI (AMT) FASC
IJBLS Editor in Chief

Over the last decade health care has seen tremendous advancements in technology that is changing the way patients are diagnosed and treated. The SARS-CoV-2 pandemic, along with the increased cases in Monkey Pox across the globe have brought significant attention to the laboratory and the need for highly skilled professionals.

Molecular diagnostics has provided a means to rapidly identify infectious diseases such as SARS-CoV-2 to improve diagnosis and treatment, but also to monitor transmission and implement preventative measures. But molecular diagnostics is more than a faster way to diagnose infectious diseases. Molecular technology has provided medicine with a means to monitor the progression of cancer and cancer treatment, develop vaccines, and genetically alter cell populations to create advanced therapeutic options that are individualized for patient treatment.

This edition of the journal provides a variety of insights into the challenges that face educating laboratory science professionals to meet the needs of a decreasing global workforce as well as new pedagogical methods. Changes in education and clinical experiences are needed to meet the shortage of professionals, but also to embrace the use of new technology and training of advanced practice laboratory professionals.

In addition to educational challenges, the articles highlight some of the technological advances that are evident in the use of molecular diagnostics and research methods that advance our understanding of complex diseases. This technology is rapidly changing and will continue to expand the impact laboratory science professionals have on medical care beyond diagnostics. Now is the time to embrace the future, and utilize new ideas to educate the future professionals that will be equipped to meet these challenges.

Sincerely, IJBLS Editor in Chief,

Patricia Tille Ph.D. MLS(ASCP) AHI(AMT) FACSc

Editorial

Handling Rejection

Hassan A. Aziz PhD, FACSs, MLS(ASCP)^{cm}
Associate Editor of Education and Administration

No one likes to be rejected whether in a personal relationship or for a professional employment opportunity. While I am not an expert in providing advice for broken personal relationships, I can shed some light on handling professional rejection.

Let's face it, for any job opening, many people will apply, but only one will get the job. It does not mean you are a bad person or not qualified for the job. It simply means the one who received the offer was the most fit for the job in the eyes of the employer. The way you handle rejection is critical in your professional career.

Many rejected candidates behave in a dreadfully unprofessional manner when they hear the "bad" news. Never burn any bridges. The lab professional community is very small, and people tend to talk. Don't add a bad label to your name where you become undesirable, regardless of your professional credentials. By acting unprofessionally, you are affirming to the employer that they have made the right decision by selecting another candidate and not hiring you.

It is believed that one's true character emerges during periods of diversity. You need to control your emotions. Instead of responding in a negative and unpleasant way to the "bad" news, consider saying: "I am disappointed because I was really excited about this position. I am extremely interested in future opportunities with your organization." Be sincere about it and show interest. You will stay in the picture and when the time comes for the organization to hire a new person, you will be first in the minds of the hiring managers.

As a candidate to an opening, you are just that. You are not guaranteed a job even if you feel you have done outstanding performance during the interview process. Others may have done better. Don't take rejection in a childish way. Threatening to suit or to take legal action is silly. Demanding for specifics on the qualifications of the hired person is absurd. Intimidating to call someone with influence is ridiculous.

After an interview, it is acceptable to contact the hiring managers with a letter, email or a phone call. However, be courteous! Show your thoughtfulness and professionalism and you will be remembered by leaving a positive impact.

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Editorial

Training Biomedical Laboratory Scientist Students for Future Challenges

Ann-Kristin Tveten, Ph.D.

Biomedical laboratory scientists (BLS) across the world have spent the last decade adapting to an increasing number of emerging infectious diseases and technological advanced for new diagnostic tools. In 2015, global travelers were warned against Zika virus, and since than there have been back-to-back challenges like reoccurring Ebola outbreaks, measles spreading across Europe and the global corona pandemic combined with the recent monkey pox outbreak. The corona pandemic has given laboratories rapid access to advanced diagnostic methods and enforced safety procedures that differ from the routine procedures in the laboratory and are usually not included in most biomedical laboratory scientist educational programs.

Systematically learning with simulation as a pedagogical method could be one approach to include training outside routine procedures and is frequently used for training nurses and medical doctors. For instance, new cutting-edge surgery techniques are usually practiced with both virtual and physical simulations. Virtual simulators with high resolution and haptic feedback provide a good foundation for learning and failing in safe environments. Physical simulations allow groups of staff from different professions to train together to optimize patient care. Simulations tend to focus on training multiple skills simultaneously, such as performing a technical procedure while communicating with others, monitoring the work environment, and handling challenges that occur.

Looking at biomedical laboratory educations, students get a lot of practical training in laboratories and in hospitals, but learning outcomes focus on routine procedures and routine tasks. Some aspects of the profession are not allowed or even possible to include in the educational curriculum. This is where simulation can contribute into BLS education, to be able to include aspects of the profession that otherwise would be impossible to teach. Nurses and medical doctors use simulator manikins to learn e.g resuscitation, in similar ways that BLS could use manikins to draw blood. For more advanced training, virtual simulations can be used to test procedures for handling samples containing potential deadly viruses or highly contagious patients.

Roleplay is also a common simulation-based training method that BLS students could use for training on blood sampling from patients with contagious diseases, airborne infections and other similar situations that do not occur on a regular basis but require a different approach than the average routine sampling or analysis. Students can get new experiences from trying

to perform blood sampling in full protective gear or wearing protective gear while trying to analyse a sample in a level 2 safety bench without breaching security protocols. These aspects are rather easy to include in BLS curriculum.

Another important aspect of simulation-based training is the possibility to perform errors in procedures and be given the opportunity to reflect on why an error occurred and how to avoid that in the future. In addition, laboratory training in BLS education is usually in low stress environments with little or no disturbances, while hospital labs can be rather busy and noisy. Simulation methods often describe how to build layers into the training to include skills that BLS students could need later in their professional life. The different aspects of soft skills, like communication, self-awareness, cross disciplinary interactions, and reflection can help build resilience for BLS students and develop their professional identity. Simulation is also a good basis for lifelong learning.

Medicine and nursing education provides extensive literature that describes design, planning, execution, and performance. A literature search reveals that it is not as common to systematically use simulation as a pedagogical method for BLS students and disseminate the results or performance. However, there are some scientific articles available from BLS education that indicate simulation is becoming more systematically used for training BLS students. Many may use simulation methods in the curriculum without realizing the huge potential there is for added learning or training of soft skills. The more literature that becomes available the more we can learn across academic institutions. BLS students are facing challenges that we do not know about today, which is why educational methods could help prepare students for unpredictable situations and how to use their overall knowledge to handle the unknown.

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Editorial: Laboratory spotlight

A Laboratory Professional's Identity as a United States Public Health Service Officer

Lt. Cmdr. David Hamilton, MLS (ASCP)

Did you know there are eight uniformed services in the United States? Six of those are made up of the military branches (Army, Navy, Air Force, Marines, Coast Guard, and Space Force) and the seventh is the National Oceanic and Atmospheric Administration Commissioned Officer Corps (NOAA Corps). The last is the Commissioned Corps of the U.S. Public Health Service (USPHS), known as *America's Health Responders*.

On July 16, 1798, President John Adams signed an act passed by the Fifth Congress that required healthcare for merchant seamen, initiating Public Health Service.[1] Public healthcare needs progressed over the next several decades and on January 4, 1889, the U.S. Public Health Service was created to fill shortages in the Marine Hospital Service, which in 1912 was renamed to Public Health Service.[1] Life expectancy of the public has increased over 30 years since its creation. Protecting, promoting, and advancing the health and safety of our nation is the mission of USPHS Commissioned Corps.[1]

The USPHS Commissioned Corps, led by the Assistant Secretary for Health and U.S. Surgeon General, is currently comprised of over 6,000+ healthcare professionals dedicated to serve at the forefront in defending threats against the public health of this nation.[1] U.S. Public Health Service active-duty officers are also ready for responding to public health emergencies. "As the cornerstone of U.S. crisis response, officers deploy to natural disasters, disease outbreaks, global public health emergencies, and serve on humanitarian assistance missions."[1]

U.S. Public Health Service officer healthcare specialties include physicians, dentists, nurses, pharmacists, clinical and rehabilitation therapists, dieticians, engineers, health and environmental health officers, scientists, and veterinarians.[1] These U.S. Public Health Service officers are found in all 50 states and other foreign assignments, working for 20+ federal departments or agencies (e.g., Food and Drug Administration, Centers for Disease Control and Federal Bureau of Prisons).[1]

The Federal Bureau of Prisons (BOP) is responsible for the custody and care of over 158,000 inmates in 122 facilities across the U.S.[2] The BOP employs healthcare professionals, civilian and U.S. Public Health Service officers, to provide medical, dental, and mental health services.[2] Acute and chronic health conditions are treated within ambulatory care units as well as medical referral centers (MRC).[2]

There are six MRC facilities who operate onsite College of American Pathologists (CAP) clinical laboratories. Each laboratory employs a combination of civil service and U.S. Public Health Service officer medical laboratory scientists (MLS). Laboratory professionals serve in various roles such as laboratory managers, MLS generalists or specialists, and healthcare administrators for this agency. I spent 8+ years working as a BOP civilian MLS employee at the Federal Medical Center (FMC) in Lexington, Kentucky where approximately 1,200 to 1,500 inmates are housed. This laboratory performed testing in hematology, general chemistry, urinalysis, and immunology. Each of the laboratory professionals have daily interactions with inmate patients, which included performing phlebotomy. While providing care for inmate patients can be challenging due to security policies, laboratory testing was performed according to community standards, including Clinical Laboratory Improvement Amendments (CLIA) law and College of American Pathology (CAP) accreditation guidelines.

As a civilian employee, I worked side by side with U.S. Public Health Service officers in and out of the laboratory. I worked as a generalist approximately five years until being promoted to laboratory manager. Collaborating with those officers made an impact on my professional life that led to a decision to convert to uniformed service. On October 5, 2015, I officially began my career as a U.S. Public Health Service officer as the laboratory manager at FMC Lexington. While my job duties as the laboratory manager did not change, the role I played and the mission it filled was much different. My responsibilities were no longer confined inside the walls of the laboratory but had expanded to the role of improving health of the underserved and vulnerable populations throughout the U.S.

Soon after beginning my career in the USPHS, the BOP promoted me to fill the role of National Laboratory Administrator. This position is centered entirely on the administrative scope of practice functions such as implementing economically efficient strategies for operating the six diagnostic clinical laboratories, determining what services will be provided, designing quality management plans, evaluating the utilization of laboratory tests, and providing consultation for agency leadership. Having a national impact through this administrative position, aligned perfectly with the mission of the USPHS Commissioned Corps, where healthcare is being delivered to an underserved and vulnerable population of patients in the BOP.

One of my responsibilities during the Covid-19 pandemic was to procure laboratory testing resources to meet the demands of the pandemic. In the beginning stages of the pandemic, my office was inundated with “Covid-19 Test Kits” claiming to have a reliable methodology for detecting the virus. It was my responsibility to determine if the Food and Drug Administration (FDA) had issued an Emergency Use Authorization (EUA) for each kit and examine their reliability. Through these reviews, national contracts were obtained to perform Covid-19 testing. Covid-19 presented some unique challenges for every healthcare provider, but the challenges were magnified because of the congregated settings found in prisons. To effectively navigate congregated settings during a pandemic, a tremendous amount of testing resources was required. While the situation presented overwhelming moments at times, the satisfaction of leading the charge for making testing available on such a large scale, removed any negative emotions.

It is an honor to serve my country as a laboratory professional. As a U.S. Public Health Service officer, I found a purpose in my career that was not present prior to accepting my commission. Whether my role expands in the BOP or takes another direction for a different agency, the commitment will remain the same. “Our Mission is to protect, promote and advance the health and safety of our nation.”[1] “In Officio Salutis” (In Service of Health).[1]

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Review article

A Modified Approach to Medical Laboratory Science Clinical Experiences in a Health System in the United States

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One of the most significant challenges contributing to the increased vacancy rates and workforce shortages of Medical Laboratory Scientists (MLS) in the United States is the inability of students to procure a clinical site or clinical programs for training and practical experience. This is due to shortages of practicing MLS professionals required to maintain internship programs and provide training. Baby boomer retirements, laboratory program hiatus, and, more recently, COVID-19 have escalated employee demands and intensified resignations, career changes, and premature retirements within the clinical environment. Elevating vacancy rates within organizations has sparked an evaluation of barriers and pathways to support modification of existing MLS training programs. As a result, this paper provides strategies to incorporate additional laboratories and use best practices in laboratory simulation and online learning to increase student opportunities for clinical training within a hospital or health care organization. Synchronizing didactic lectures and clinical instruction, using online Problem-Based Learning (PBL) and weekly Team-Based Learning (TBL) can eliminate the need for rigorous one-on-one clinical rotations. Health care organizations and clinical laboratories must collectively address current and future needs in the MLS workforce. Pedagogical changes are needed to increase the number of student opportunities for training within clinical laboratories and strong support to establish a framework to modify approach to MLS clinical site training.

Key words: MLS, Student, Vacancy rates, Clinical Laboratory, Clinical Experiences.

Introduction

Medical laboratory scientists (MLS) have an immeasurable role in providing vital information for managing patient diagnosis and treatment by conducting diagnostic laboratory testing. Medical laboratory professionals deliver a staggering 13 billion diagnostic tests annually in the United States to physicians who will make consequential patient care decisions with the information.[1] As one of the highest volume medical activities influencing nearly 2/3rd of the decisions made by physicians, clinical laboratory testing can save future costs, time, and even patients' lives when used for early detection and disease prevention.[2] Without medical laboratory professionals, physicians would be blindly treating the patient.

MLS are considered healthcare detectives, yet despite the importance of the work, they are also considered a "hidden profession."

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The COVID-19 pandemic brought the Clinical Laboratory Scientist (CLS) profession to the public eye, but it also exacerbated well-known concerns, including understaffing, increased workload, and work-life balance issues.[3] Clinical laboratorians had to pivot quickly, validate new analyzers, and adapt to unexpected conditions, including lack of personal protective equipment shortages (PPE), social distance requirements, and unprecedented numbers of COVID-19 testing while maintaining the regular workflow.[4]

The nation faces a growing and critical shortage of qualified medical laboratory staff in clinical laboratories. The U.S Department of Labor, Bureau of Labor Statistics projects an increase in demand for MLS of 11 percent from 2020 to 2030, faster than the average for all occupations.[5] The number of physicians and other healthcare providers requiring specimen analysis for overall health and diagnostics creates a demand requiring skill and competence. Unfortunately, there are not enough qualified medical laboratory professionals practicing in the United States to sustain the testing necessary for 330 million Americans. The scarcity of certified MLS impedes the ability of clinical laboratories to meet the growing demands of patient testing and could present difficulties for patient care and well-being. On average, about 25,900 openings for MLS and medical laboratory technicians (MLTs) are projected each year, resulting from a departure in the labor force due to retirements and, more recently, resignations and increased absences experienced during the COVID-19 pandemic.[5]

In addition to the increased demand for medical laboratory professionals, there are insufficient prospects for the onsite clinical practical experience required to prepare MLS trainees. Clinical experience is an integral part of any medical laboratory science training program. In fact, California defines clinical training as "Practical experience means hands-on, direct work experience in clinical laboratory science techniques on real patients in a clinical laboratory certified by the Clinical Laboratory Improvement Amendments of 1988 (CLIA)."[6] MLS students receive instruction under direct and constant observation and evaluation by practicing MLS. However, increased vacancies in clinical laboratories prevent organizations from offering the practical clinical experiences required for proper training and education of MLS students nationwide.

In addition to increased vacancies within clinical laboratories, NAACLS-accredited MLS programs have minimally increased in the last ten years, from 226 in 2011 to 239 in 2021, leaving laboratory leaders wondering if MLS programs can sustain the projected growth rate for medical laboratory science professionals.[7,8,9] Concerned stakeholders, including universities, clinical affiliates, employers, and professional organizations, are encouraged to take action through collaboration and strategizing to satisfy qualified medical laboratory professional demands in the years to come.

All clinical laboratory organizations, whether they are large health systems or smaller clinics need to consider adding training rotations to compliment existing laboratory sites to share in the training of new laboratory professionals. Laboratories could add additional students to the MLS training programs and increase the potential candidates available for hire each year. Creating a modified approach to MLS clinical site training coupled with changes in pedagogical delivery of didactic content, has the potential to expand to include the smaller facilities, and using best practices in laboratory simulation would eliminate the need for rigorous one-on-one clinical rotations and alleviate the additional burden on current laboratory professionals in training roles.

Background

Aging workforce

As baby boomers retire, the clinical laboratory loses many experienced and well-developed

professionals. According to the American Society for Clinical Pathology (ASCP) vacancy surveys, retirement rates for those retiring in the next five years are at their highest levels across multiple clinical laboratory departments. The blood bank anticipates a staff member reduction of 13.0% and 24.3% for supervisors, core laboratory retirements for staff are highest among the departments surveyed at 15.1% and 32.1% for supervisors, and the microbiology staff retirement rate is 12.8% and 30.9% supervisors (Table 1).[10,11]

Many baby boomer laboratorians joined the profession before training program closures began in the late 1990s. These experienced individuals have worked in the field extensively and serve as technical experts. Long-time laboratorians easily recall performing manual techniques to determine analyte values or identify bacteria. High-throughput laboratories often use closed system automation for testing analysis, but the principles behind the testing are based on manual methodology. This practical experience gained through hands-on experience is rarely practiced in the laboratory today. The loss of medical laboratory professionals retiring from clinical laboratories not only diminishes the number of employees in the laboratories but also removes an abundance of knowledge and experience. The demand for laboratory testing will also increase as the population increases and ages.

Vacancy Rates

Long before the Covid-19 pandemic, laboratories nationwide were experiencing staffing challenges. In a 2015 article by Kimberly Scott for the American Association of Clinical Chemistry (AACC), she stated, "The shortage of qualified laboratory professionals is not a new story; it is also one that won't go away." This statement continues to ring true today.[12] A 2012 survey conducted by ASCP identified vacancy rates for clinical laboratory personnel across the nation at 6.0%, with 9.0% of the employees expected to retire within 24 months.[10] Responders to the 2012 survey indicated "aging labor force, improvement in science and technology, and laboratory program closure" as the causative factors in the laboratory labor force status. As growing demands for medical care increase with an aging population and medical care advances, laboratory test volumes grow. Inexperienced medical laboratory professionals new to the job and substandard financial earnings compared to other health care professionals contributed to the shortage of well-trained, competent personnel in 2012. Similarly, today, limited program availability and the retiring workforce create staffing shortages that are difficult to fill.

Medical laboratory science vacancies have intensified due to disruption in the workforce caused by the COVID-19 pandemic and expected retirements. Declining numbers of students entering accredited education programs increased demands on employees, competitive salaries and sign-on bonuses at various laboratories, and staff burnout have exacerbated resignations, career changes, and premature retirements on top of the baby boomer generation retirements.[3]

Although COVID-19 increased medical laboratory professionals' visibility, it has negatively impacted MLS education primarily

Table 1: Percentage of Staff (nonsupervisory) and Supervisory Retirements rates, 2020 and 2012.

	Retirement Rate, %					
	Staff (nonsupervisory)			Supervisory		
Department	2020	2012	Δ	2020	2012	Δ
Blood bank	13.0	7.0	6.0 ↑	24.3	8.0	16.3 ↑
Core lab	15.1	6.0	9.1 ↑	32.1	24.0	8.1 ↑
Microbiology	12.8	9.0	3.8 ↑	30.9	10.0	20.9 ↑

by halting or delaying clinical rotations. Students were limited in laboratory rotations, sent home for safety precautions and social distancing, and sites stopped taking students during the pandemic.[13] Students could not get the "practical experience" required to qualify for state licensing where required, worsening the increasing staff vacancies in areas such as California. It appears the pandemic has already accelerated future shortages in the CLS field. Laboratory professionals must prioritize actions to define solutions and the next steps to strengthen the medical laboratory professional workforce.[3]

Cost of Education

Competition for medical laboratory personnel has intensified over the last several years, and staff resignation rates are beginning to be an increasing problem. To fill vacancies nationwide, some laboratories are hiring noncertified staff. This is not an option for regulated Clinical Laboratory Improvement Amendments (CLIA) laboratories. CLIA is responsible for regulating diagnostic testing before use on human blood samples and requires clinical laboratories to be certified by the Center for Medicare and Medicaid Services (CMS).[14] Hiring noncertified laboratory personnel is not a legal option in some licensure states such as California, nor is it an option that some laboratories wish to consider. An Oklahoma City laboratory conducted a study and found there were increased errors and lengthier training times required for employees lacking certification compared to those with certification. Additionally, these employees failed to recognize critical results "because they don't know the clinical difference in a test." These problems required additional time allocation and financial resources.[15]

Similarly, training additional students in a laboratory setting raises concerns that these actions may negatively affect productivity. But investing time in educating students within any organization can assist with a significant return on investment. The cost to recruit a new employee not trained internally requires factoring in evaluation and authorization to replace the vacancies, interview time and lost productivity for supervision, time to process newly hired applicants, and orientation and training time.[16] Students educated within a clinical laboratory organization are future pretrained employees who will require less instruction during the probationary period; this is where the organization can save money in the long run.[12]

Training programs

In 1983 approximately 9000 individuals graduated from accredited MLS programs; in 1992, the numbers decreased to 5760.[17] The percentage of graduating laboratory professsionals in 2019 diminished to 3663, 36 percent compared to almost 30 years ago, but the population in the United States increased by more than 20 percent.[3] Over the last 19 years, MLS training programs have decreased by 50%. According to the NAACLS, MLS programs have gone from 468 in 2002 to 239 in 2021.[8,9]

The decreased number of MLS training programs has prevented qualified individuals from being accepted into accredited training programs. Prospective students often must wait a whole year to reapply. Limited training spots create a highly stressful environment for students and clinical affiliates. Developing strategies to address these concerns must be at the forefront of conversations in the field and requires a collective effort by interested parties at all levels within the clinical laboratory organizations. Many college graduates in biology and science are still unfamiliar with the profession and the pathways leading to laboratory careers. Current literature states that enhancing the MLS profession's visibility through outreach to primary schools, high schools, professional networks, and college campuses can encourage recruitment into the field.[3] But where will these students train? The shortages of clinical training opportunities continue to limit the recruitment route and the future success of individuals in the profession.[3]

The current laboratory personnel shortages continue to negatively impact staff work-life balance, morale, and retention. A satisfaction study by the ASCP in 2020

identified participants had high job fulfillment but only a fair work-life balance. The main reasons for dissatisfaction were elevated job-related stress, burnout, additional responsebilities, loss of work schedule flexibility, and understaffing.[17] More than half (59.1%) of the survey respondents reported feeling inadequately compensated for their work. The objective of developing a modified MLS training program is to address the current vacancy increase and expected growth rate in MLS shortages over the next decade.

Curriculum design

Regulatory Requirements

NAACLS recognizes and accredits hospital affiliated university programs across the country to meet the established standards in clinical laboratory sciences. NAACLS standards serve to develop, maintain, and promote quality, provide recognition, and assist in developing and evaluating medical laboratory science programs.[19] Clinical site responsebilities include having at least one medical laboratory professional liaison per site responsible for coordinating clinical instruction and maintaining communication with the university program director. If a program is a stand alone hospital-based NAACLS accredited program, no clinical liaison is needed.

NAACLS standards require a clinical curriculum to include clinical chemistry, hematology/hemostasis, immunology, transfusion medicine, microbiology, urine, body fluid analysis, molecular diagnostics and laboratory operations.[19] The curriculum at each clinical site must address pre-analytical, analytical, and post-analytic components. Additional topics include safety, regulations and standards, and good communication to serve the needs of the service and enable students to achieve entry-level competencies in each discipline. Although NAACLS standards require a wide variety of curriculum topics for instruction, there is no documentation of specific timelines or rotational obligations. Clinical laboratories or university program must also meet several minimum requirements to train MLS students. These requirements include adequate space and equipment, and minimum instructor to student ratios as determined by the clinical laboratory or university based program administration.

Current Training Programs

Many hospital-based and some university programs continue to adhere to outdated pedagogical training schedules. One such laboratory, Los Angeles County Department of Health Service (LAC-DHS) laboratories is required to strictly adhere to California state requirements, and each student receives 52 weeks of individualized training at the three hospitals. Student training mimics the training program required for a newly hired MLS professional, except students are not allowed to perform patient work.[20] Students are paired with an MLS for 5-6 hours a day for four days a week. Trainers prepare them to think critically, navigate the laboratory information system (LIS), and practice manual methods used in clinical patient care. Students observe licensed MLS professionals verify patient results, and as time permits, they are provided with step-by-step instructions to resolve test result abnormalities and inconsistencies that require investigation. Students do not train together as they rotate throughout the clinical rotations. They each work independently with a seasoned MLS professional. It has always been this way, and it worked. For years LAC-DHS, like many other laboratory science organizations took pride in the ability to train students one-on-one and devote dedicated time to them. The ability to do a quality job with the increase in vacancies makes this approach challenging and unsustainable, and it affects the value of the practical experience required to prepare MLS trainees to meet professional requirements.

Hospital or clinically based NAACLS accredited programs may provide online or face-to-face didactic lectures one day per week or half-days every day, based on the program design. Each program is unique depending on clinical rotation, classroom and

instructor availability. The lectures do not always coincide with the clinical rotations for every student, and this asynchrony creates difficulties for students unable to connect technical information with practical experience. Synchronizing didactic lectures and clinical instruction may increase student engagement with didactic material and alleviate the repetition of technical explanations from clinical instructors, allowing them to focus on workflow tasks. This would likely require programs to utilize on-line learning management systems to provide lectures simultaneously for all students independent of their individualized clinical rotations. Upon completing the clinical training and didactic lectures, students are qualified to take the Board of Certification (BOC) of the ASCP national certification examination.[22]

In addition to implementing online instruction many clinical laboratories rely on a single large laboratory to train the MLS students in all the major disciplines required by NAACLS. Training students independently in a single medical center's core laboratory, composed of chemistry, hematology, urinalysis, and coagulation, occupies a major part of the student clinical training. This leaves little time for the core laboratory staff to onboard and instruct new hires, perform instrument validations and computer upgrades, prepare for inspections, and implement new projects without feeling overwhelmed and anxious. Utilizing additional smaller affiliated laboratories that perform high complexity testing within the core disciplines, would provide an opportunity to expand clinical experience training. Each laboratory generally has the technical infrastructure to cover the three phases of laboratory testing in biochemistry, hematology, coagulation and urinalysis to support MLS students. Implementing training on a broad scale across multiple laboratories must be tested and designed properly to determine whether this structure can provide quality MLS training to more students in each health system. No one size fits all.

Additional pedagogical innovations

MLS programs aim to train students to master theory, gain professional knowledge, and develop skills in clinical laboratory science.[23] Didactic material establishes a foundation for the clinical laboratory experience to build on. Clinical rotations teach what one can only learn in the clinical setting, such as onsite workflow managing numerous specimens, multitasking, instrument functionality, maintenance and automation, regulatory compliance, and documentation of consistent, official records.[21]

Moreover, clinical laboratory affiliates are focused on hiring new graduates and have invested time in training. Clinical laboratories can overcome laboratory shortages by modifying current MLS training programs and increasing the number of students accepted within each organization annually. Doing so will provide a larger pool of qualified candidates to fill vacancies.

Innovative Solutions

A recent study in a clinical pathology course explored the impact of blended learning, the combination of an online classroom and in-person learning, and its acceptance as a form of education in the discipline. It determined that there was astronomical acceptance by students and faculty alike and above-average achievement compared to conventional educational methods alone.[24] The pandemic altered educational platforms. In-person learning quickly transitioned to online audiences and has become widely adopted as a preferred method across college and university campuses. Why should this be any different for all MLS programs including hospital -based?[25] With technological advancements, online learning, and engagement through a computer has become more prevalent. As demands increase for educational programs and clinical sites to educate more MLS students with scarcer resources, online

education becomes a more appealing environment for educating future professionals. Synchronous and asynchronous virtual learning can be an effective educational method for delivering clinical lessons and, if constructed clearly and with detail, can provide lasting and consistent instruction.[26]

In fact, some universities have already changed clinical rotation timelines and are focusing on "getting students comfortable working in the hospital laboratory setting."[27] The University of Minnesota's MLS program reduced the time MLS students spend on clinical rotations from 22 to 12 weeks. The MLS accelerated program taught at South Dakota State University uses a combination of online, on-campus, and clinical courses in a 16-month program. This allows students to complete coursework faster than in traditional on-campus programs. Class sizes are as large as 24 students, and ASCP Board examination pass rates between 2018 and 2021 were 95%, respectively.[28]

These programs' success and ability to increase seats at clinical sites rely on exposure to testing methods or mock patient samples at the university using tabletop instrumentation similarly to those found in clinical laboratories. Students experience hands-on opportunities with laboratory information systems, digital microscopy, and specimen results that mimic real-life scenarios in simulation laboratories with detailed rubrics that grade students on turnaround time, accuracy, and reporting of critical values.[21] Simulation laboratories are not meant to replace in-person clinical training but may be used to help meet clinical hour requirements in these scenarios.[29]

Technology Based Learning Strategies

Clinical diagnostic laboratories must work together to address the challenge of significant MLS vacancies. To increase the number of MLS students trained annually, laboratory leadership should evaluate the current curriculum and determine how to best prepare students to master skills and perform clinical tasks skillfully with existing resources. By focusing on the purpose of clinical rotations and creating new models of education using online resources and affiliations with sister facilities, the organizations can successfully train more students annually.

Programs can easily create online tutorials as well as other educational materials using technological advancements in the online learning environment. Online tutorials may consist of recorded experimental or practical work supporting and enhancing learning and teaching. Implementing effective online learning that includes problem-based learning (PBL), self-study modules, and digital resources allows students to visualize clinical concepts in action, creating favorable outcomes. Online learning provides students with "manageable and effective access to a wider variety and greater quantity of information. These platforms accommodate students with various learning needs, promote interest in instruction by using multiple methodologies and nurture knowledge of clinical laboratory science by seeing processes completed correctly by the instructor that can be re-watched multiple times.[27] Researchers have identified no significant difference in MLS trainees' test scores and the ASCP Board of Registry certification scores for online learning students compared to traditional learning.[25]

Decreasing clinical rotations and focusing on the student "getting comfortable in a clinical laboratory" is a desirable option to train an increased number of students annually. However, it must be noted that some licensure states are still severely limited to address the shortage based on state regulations. Presently, California requires a bachelor's degree, with specific course requirements and a year of internship, to become eligible to take the ASCP MLS board exam to become both California licensed and nationally certified. The 52-week practical training requirements limit programs from altering the number of weeks a trainee must dedicate to the required subject by receiving hands-on direct work experience with actual patient samples. However, there is no clear hourly

requirement per week documented. The California Code of Regulations, last updated over half a century ago, needs modernizing. If not taken seriously, organized strategies to address the MLS workforce shortages will not be enough to reverse California's systemic problem based on limited clinical training capacity.[30]

The idea of the one-on-one teaching program historically offered through many clinical laboratory training programs is attractive to many MLS trainee candidates. Applying clinical skills in a laboratory environment can be challenging for students, and individualized instruction tailored to their learning style provides a platform where they can succeed. For the MLS instructor, this design is also appealing. Instructing one student at a time is less overwhelming and allows for a relationship to build with a potential future employee. Students have varying clinical skills when entering any MLS program, so with only one student to consider, the trainer can closely observe the students' strengths and weaknesses and has the freedom to modify the pace of instruction, accordingly, making this platform productive and meaningful.

Historically each student is generally paired with a practicing MLS professional for practical experience during the week and varies from four to six hours per day. Instructors review theory, and standard operating procedures, demonstrate laboratory skills and then observe the students performing similar actions on mock patient samples. Students observe the verification of patient samples and wait for unusual specimens to surface for more detailed instruction. More recently, one on one instruction has become nearly impossible to accomplish. With the shortage of laboratory professionals, students no longer get the sought-after individualized attention from the MLS trainer, who must multitask and cover multiple laboratory workbenches. Students spend more time reviewing standard operating procedures independently and observing the MLS perform patient care. There are fewer opportunities for MLS instructors to delve into abnormal patient sample workups and explanations.

With the inability to manage one on one training in the clinical laboratory, how can more students be added to the training program as the generation of baby boomers retire and with the persistent shortage of MLS and supervisors in the laboratory. An approach that, in recent years, has gained popularity in medical education is team-based learning (TBL).[31] TBL is an active collaborative approach to small group teachings and has been successfully used across numerous STEM fields (Science, Technology, Engineering, and Mathematics).[32] TBL is already used among laboratory professionals with colleagues and through interprofessional learning during communication with nurses, physicians, and pharmacists. This platform enhances critical thinking, communication skills, and active learning and would benefit students by involving them in scenarios encountered regularly in the laboratory.[33] Clinical sites may already be using a similar platform. Introducing a method for standardized clinical teaching and collaboratively education through the use of a team-based approach will eliminate many of the redundancies in processes and workforce utilization during the clinical experience.

A study on TBL in medical schools held sessions once per week for two hours, where before class, students were given required reading and pre-recorded lectures. Individual readiness was assessed using a multiple-choice quiz at the beginning of the session, followed by team-based clinical problem-solving using open-ended responses. Immediate feedback sessions, promoting discussions, and opportunities to challenge answers were facilitated by instructors, who then concluded the session by summarizing the assignment and providing key take-home messages. The TBL structure was more conducive to learning, remained student-centered, and generated positive outcomes.[31]

TBL does require facilitation from an educator. With the current clinical training structure performed in many hospitals and other health care based programs, educators may spend 20 or more hours per week with each student, collectively, and potentially adding up to over 100 hours per week discussing the same purpose of testing, analytic platforms, problem-solving, and operating procedures with individual students. One review session, face-to-face session of two hours per week with a single instructor using TBL across the student cohort can save hundreds of hours of manpower and alleviate redundancies. In turn, this would lessen the burden on laboratory staffing, as they work with each student for several hours waiting for unusual patient samples to provide opportunities for clinical problem-solving. MLS professionals could instead spend fewer hours with students providing focused, practical experiences.

The use of TBL across a student cohort would also support the addition of other smaller laboratory participation in the hands-on, direct clinical experiences. The education of analyte testing, analytic platforms, problem-solving, and operating procedures would be identical for each student. Time spent in the clinical rotations would focus on content and processes not included in the TBL sessions and didactic lectures, such as laboratory workflow, handling of specimens and multitasking, use of automation, adherence to regulatory compliance, and lab safety and record keeping.[21]

Clinical rotations are expected to provide work experience and evaluate competency in the students' ability to understand and perform laboratory tasks. Students are prohibited from verifying patient results, and training sites should not expect them to be fully proficient in these actions. Technological advancements have decreased the time required to achieve skill levels expected of MLS students by streamlining the preanalytic phase of specimen handling and eliminating labor-intensive and time-consuming manual testing. Standardization of instrumentation, operating procedures, and processes at clinical laboratory training sites would allow the additional laboratories to participate in rotational training and create room for additional students. Reaching proficiency in laboratory tasks for the students at each individual laboratory as a new employee will require site specific in-depth training in each department.

Conclusion and implications

Laboratory shortages are a reality at medical centers nationwide. Many qualified candidates are eligible to enter MLS training programs across the country. Procuring a clinical site for practical experience is one of the biggest challenges not only for hospital or health care based programs but also university programs, mainly due to shortages of practicing MLS professionals needed to maintain internship programs and provide training. This clearly affects the pipeline to provide future laboratory professionals.[34] Current program enrollments cannot keep up with the increasing demand for vacancy rates as they approach critical levels. Clinical laboratories must address and assess the ability to accept and train additional students into the existing MLS training programs.

Clinical laboratories could easily double the number of students in MLS programs by modifying current practices that include synchronizing didactic lectures with clinical instruction, using PBL online platforms and weekly TBL, and utilizing all high-complexity core laboratories to provide the clinical experience required for student training. Transitioning from what used to be to what needs to be will not be without challenges as laboratorians face increasing time constraints and demands. Additional studies and assessments are required to understand best practices with online learning, PBL, and TBL and how the modified training will align with each required task. The use of additional laboratories will need guidance and preparation in

coordinating clinical instruction and maintaining communication with the MLS program director per NAACLS guidelines.

Discussing the future of clinical training with various laboratory managers, identified a desire to increase the number of trainees and support to establish changes to program practices. One lab manager even suggested "starting early to form the framework."[35] Laboratory leaders must develop new ways of providing a personalized, self-directed learning experience if clinical diagnostic laboratories wish to maintain the success achieved through one-on-one training. Collaboratively, clinical organizations can develop best practices in MLS training across affiliated laboratories, eliminate the need for rigorous one-on-one clinical rotations, and alleviate the burden on staff in training roles. Providing an opportunity for more students to secure a spot in a clinical site for practical experience and resolving the biggest problem facing the training of future MLS professionals. Clinical diagnostic laboratories will also need to improve recruitment of well-qualified, knowledgeable MLS candidates for the future of the profession. Success also relies on leadership support, a willingness to modify current practices, and the ability to reflect expectations of the profession and prepare students for successful entry into the clinical laboratory environment.

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Review article

Advancements in Targeted Molecular Therapy for Human Papillomavirus (HPV) Related Cancer

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Human papillomavirus (HPV) is a common sexually transmitted disease among both men and women. High risk cases can quickly develop into cancer, most frequently in the cervix and oropharynx. Standard treatment options include surgery and chemotherapy both of which are painful, hard on the body, and can leave the patient with long term side effects. Unlike traditional therapy methods, molecular targeted therapy focuses specifically on molecular changes making it more effective, highly specific, and more tolerable than more traditional methods. Molecular targeted therapy has shown promising results for various types of cancer. Recent developments for HPV specific cases have led to some exciting advancements in precision medicine.

Cetuximab and gefitinib are two recently developed molecular targeted therapy drugs that target epidermal growth factor receptors (EGFR) to deactivate molecular pathways responsible for cancer growth. Both drugs are proven to be safe and effective therapy options that can improve the patient's overall survival and decrease disease recurrence. However, drug resistance remains problematic for patients using molecular targeted therapy. A common solution is combining molecular targeted therapy with additional options such as chemotherapy or other targeted therapies. This has the potential to eliminate drug resistance. However, there are limited target therapies available for HPV cancer. This demonstrates the need for further research and drug development for HPV related cancer cases to make further advancements.

Key words: Human papilloma virus; Cancer; Molecular targeted therapy; Molecular target identification; Oropharyngeal; Molecular detection; miRNAs; Epidermal growth factor signaling; Kinase inhibitors; Monoclonal antibodies; Cetuximab; Gefitinib; Resistance; T790M

Introduction

Human papillomavirus (HPV) is a viral infection that is primarily sexually transmitted and can lead to HPV-related cancers in both men and women. It is currently the most common sexually transmitted infection in the United States.[1] High risk HPV infections are the cause for all cases of cervical cancer and some cases of oropharyngeal cancer.[2] The HPV-16 subtype accounts for approximately 95% of HPV-positive cases of oropharyngeal cancer and subtypes HPV-16 and HPV-18 are responsible for up to 70% of the cases of cervical cancer.[3] Less

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frequent HPV genotypes that also contribute towards the development of cancer include 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82.[3] In addition, HPV is associated with multiple noncervical malignancies. These can include vulvar, vaginal, penile, anal, esophageal, and head and neck cancers.[2] The number of head and neck cancers linked to HPV, especially oropharyngeal cancer, has been progressively increasing.[1]

Cervical cancer is the most common HPV-related disease and cancer in women. HPV is classified as a sexually transmitted disease. However, it has been reported that skin-to-skin contact can be efficient enough to facilitate viral transmission. HPV is associated with a variety of clinical conditions including lesions, warts and occasionally cancer.[6] Almost 80% of males and females will test positive for an HPV infection at some point in their lifetime.[1] Many low-risk HPV infections are benign and cause lesions such as cutaneous warts on the hands, feet and anogenital regions.[4] Typically, the immune system will clear the virus, however in some cases the infection can progress into high-risk HPV and lead to precancerous changes or tumors.[1,4] The virus infects the mucocutaneous epithelium and produces viral particles in matured epithelial cells. This causes a disruption in normal cell-cycle control. The promotion of uncontrolled cell division can then lead to genetic damage.[4]

Oropharyngeal cancers have been linked to tobacco and alcohol consumption. However, recent studies show that about 70% of cancers of the oropharynx are linked to HPV infection.[1,3] Over the past 20 years in the United States, the detection of HPV related oropharyngeal tumors has increased from 16% to 73%. The oropharynx has become the most common site of HPV-related cancer, surpassing the cervix. Male predominance is evident in HPV related oropharyngeal cancer with a male-to-female ratio of about 4:1. This may correlate with a higher prevalence of oral HPV-16 infections in men opposed to women.[3] Recent studies have also investigated the differences in HPV genotype distribution in comparison to age. It was shown that there is a higher level of HPV in younger HPV-positive oropharynx cancer patients in comparison to older patients. HPV positivity in oropharynx cancer depends on the intensity of sexual exposure, explaining the lower numbers of HPV positive cancers in older patients. The reduced use of alcohol and tobacco has decreased the number of oropharynx cancer cases however, the HPV infection-related cases have begun to increase.[1]

As HPV infection has become more notable in oropharyngeal cases as well as cervical cancer, it is important that treatment options improve. Standard treatment options for patients diagnosed with HPV related cancer include surgery, chemotherapy, and local radiation therapy.[2] Patients diagnosed with cancer in the early-stages are generally treated with radiotherapy or surgery while more advanced cancer is treated with a combination of surgery and either radiotherapy or chemoradiation.[5] HPV related cervical cancer patients are often treated with radiation therapy, although some patients will require a full hysterectomy to remove the cancer.[3] However, recurrent and metastatic disease remains one of the main causes of mortality in HPV related cervical cancer.[2] Patients with early stages of HPV related oropharyngeal cancer can also be treated with surgery and radiation therapy. However, many patients with oropharyngeal cancer are not diagnosed until the cancer has reached an advanced stage. Therefore, harsher treatments are required which includes a combination of chemoradiation and surgery followed by several treatments of radiotherapy.[3] These standard treatments are often successful in eliminating cancer, however they are extremely toxic and damage other tissues and muscles in the surrounding organs. These severe side effects have led scientists to conclude that the current standard of care is over-treating patients. By restructuring some of the treatments it might be possible to achieve

comparable survival outcomes while simultaneously lowering toxicities and reducing the need for invasive surgical procedures.[5]

As new findings and approaches in molecular biology have become available, advancements in molecular targeted therapies have become an option for treating patients with HPV related cancer and are not associated with the negative long-term side effects.[5] Molecular targeted therapy is the use of drugs or other substances that target specific molecules to block the growth and spread of cancerous cells. Selecting the appropriate molecular target is essential for successful treatment. Food and Drug Administration (FDA) approved molecular target therapies have been successful in the treatment of various types of cancer.[6]

Limitations associated with targeted molecular therapy for the treatment of cancer includes the specificity of the biomarkers, and the need for a better understanding of how to implement this technology in treatment regimens.[5,6] The success of molecular targeted therapy is dependent on a patient's tumor expression of the specific biomarker used as the molecular target. Different genetic mutations occur that can be linked directly to the development of cancer. Therefore, identifying the molecular target for successful treatment can be difficult.[6] Currently, there are very few molecular target treatment methods available that are FDA-approved. This is partially due to the methods not pairing well with patients undergoing radiation. A better understanding of the biology behind HPV related cancer is needed to design newer therapeutic strategies. Targeted therapies must be safe and demonstrate an improvement in patient outcomes in comparison to the available standard treatment options such as surgery, radiotherapy or chemoradiation.[5]

Molecular targets

To develop molecular targeted therapies, it is important to consider what type of molecules may be useful for further analysis and consideration for the treatment of HPV related cancer. Growth factors, signaling molecules, cell-cycle proteins, apoptosis modulators and molecules that promote the development of new blood vessels are potential therapeutic molecular targets.[6] Molecular signaling pathways involved in the deregulation of critical molecular processes are also an option for use as a molecular target.[2] There are several items to consider when choosing a molecular target. Not every molecule in a cancer cell has the potential to be used as a target. Molecules that have been identified to function as useful targets are often embedded in the outer membranes of tumor cells. The cell membrane molecules tend to bind to therapeutic drugs and are effective molecular targets.[7]

Molecular Targets in Drug Development

Therapeutic drugs, oral or injectable, can be designed to effectively bind molecular targets present in cancer cells responsible for promoting tumor growth. The drugs can be used to prevent the formation of new blood vessels cutting off the nutrient and oxygen supply to the tumor cells resulting in cell death.[6]

In addition to the preferred location of a target molecule in the cell membrane of a tumor, a target molecule should also be present in many of the patients with a specific tumor type. The target molecule should not be present in normal tissue, otherwise use of the target may result in toxic side effects to the patient. It is also important to determine if the target molecule is associated with resistance to apoptosis, uncontrolled cell proliferation, increased cell migration, altered cellular adhesiveness, or modulation of the immune response.[7] If the molecule is not associated with toxicity or other cellular functions as noted it can then be considered a potential molecular target for therapy.

HPV Molecular Targets

Various molecules have been identified as targets for HPV treatments. The early (E) HPV proteins E1, E2, E4, E5, E6, and E7 are necessary for viral integration, replication and

transcription. E6 and E7 are considered oncoproteins and are responsible for cell proliferative signaling, deregulating cellular energies, and avoiding growth suppressors. While the primary role of the E5 protein is to avoid immune destruction. HPV E5, E6, and E7 oncoproteins are capable of altering the functions of multiple signaling pathways and inducing cervical cancer.[2] Viral E6 and E7 oncoproteins alter transcriptional control. Therefore, detection of the abundant expression of HPV *E6/E7* transcripts can be used to predict underlying cervical precancer. This correlates to the presence of high-risk HPV (HR-HPV) rather than simply viral presence.[8] E6 targets the tumor suppressor protein p53 by forming a complex with the E3 ubiquitin-protein ligase E6-associated protein (E6AP) promoting proteasomal degradation. The viral protein complex can also block transcription of the tumor-suppressor. The degradation of the p53 protein promotes viral replication leading to the accumulation of genetic mutations that result in host cellular transformation, dysplasia, and cancer.[9]

E6 and E7 also play a role in oropharyngeal HPV-related cancer. Oropharyngeal HPV-related tumors occur around the tonsils, base of the tongue and soft palate. The cancerous tumors develop when viral oncoproteins E6 and E7 are overexpressed and promote tumor progression by inactivating the *TP53* and retinoblastoma tumor suppressor genes. E7 will bind to the retinoblastoma *protein (Rb),* which will disrupt the cell cycle and initiate transcription of S-phase genes.[8] This will then stimulate the phosphoinositide 3-kinase (PI3K), protein kinase B (Akt) pathway which is a main cancer survival pathway involved in signal transduction.[2] The tumor suppressor proteins, p16 and Rb help regulate the G1 and S phase of the cell cycle. When HPV infection causes cancer, Rb becomes functionally absent and p16 is overexpressed due to the loss of negative feedback.[8] When AKT becomes phosphorylated it can lead to increased tumor progression, and drug resistance. This makes an oncogene mediated pathway such as (*PI3K*)/(*Akt*) a promising molecular target.[2]

Another potential target for both cervical and oropharyngeal cancer is micro ribonucleic acids (miRNAs). The miRNAs are small non-protein coding human RNAs that are about 18-25 nucleotides in length involved in the regulation of gene expression and messenger RNA (mRNA) translation and decay.[2,10] Human miRNAs are also involved in the regulation of cell growth, apoptosis, cell migration, and metastasis. miRNAs may be useful as biomarkers for the detection of several types of cancers including HPV. A single miRNA can alter the expression of hundreds of genes. This makes the identification of the change in expression of a specific or multiple miRNA's associated with cancer as potential targets for use in diagnostics and therapeutics.[2] The miRNA patterns are also tissue-specific which makes it easy to distinguish carcinomas from normal cells.[10]

Advancements in HPV therapy

HPV targeted molecular therapy uses agents such as tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (mAB) to block the growth of malignant cells by interfering with the essential pathways needed for the development of tumors.[11,12] TKIs are small molecular weight drugs that are orally administered. A TKI will bind a targeted sites to induce molecular changes that inhibit kinase enzyme activity. A mAB is administered via injection since the molecular size of the antibody is too large to penetrate cellular membranes. The antibodies interfere with signal transduction from the cell surface and provoke apoptosis or interfere with protein functions needed for the growth of cancer cells.[12] Targeted molecular therapies may use mABs and TKIs to target epidermal growth factor receptors (EGFR). EGFR receptors contribute to disease progression and therapy resistance in a variety of human cancers.[7]

Epidermal growth factor receptors are transmembrane glycoproteins responsible for

the regulation of cell growth and proliferation.[13] The presence of an HPV infection causes an upregulation of EGFR signaling and contributes to the progression of cancer in the cervix and oropharynx.[11] Most HPV related cancers over express EGFR on the surface of tumor cells making it a good target for molecular therapy.[14]

Advancements in molecular targeted therapy for HPV patients includes the use of the therapeutic drugs cetuximab and gefitinib. These drugs have shown promising results in clinical trials by successfully reducing tumor activity in HPV patients. Cetuximab uses mABs to bind to the extracellular domain of EGFR while gefitinib uses TKIs to bind to the intracellular kinase domain of EGFR.[14]

Cetuximab

Cetuximab is the only FDA approved targeted therapy proven to increase the overall survival of HPV related head and neck squamous cell carcinomas (HNSCC) in combination with radiotherapy.[14] Cetuximab is an injected monoclonal antibody used for oropharyngeal cancer that targets EGFR. It is also the only drug proven to treat both recurrent cancer and cancer restricted to a localized region of the body.[13,14] Cetuximab binds to the extracellular domain of the EGFR and blocks the activation of the protein receptor's intracellular domain interfering with the associated tyrosine kinase-dependent signal transduction pathway. This results in the internalization of EGFR removing the receptor from the cell surface and prevents any interaction with the protein ligands.[13] Cetuximab effectively alters the cell surface membrane structure which initiates apoptosis of tumor cells by activating natural killer (NK) cells and inducing antibody dependent cellular cytotoxicity.[11]

In addition to blocking EGFR, cetuximab blocks cellular secondary repair mechanisms dependent on the *PI3K/Akt* pathway mitogen-activated protein kinase **(**MAPK), and Janus kinase/ signal transducer and activator of transcription of the (*JAK/STAT3*) downstream signaling pathway. This causes a reduction in the capacity of the cellular DNA repair as well as decreasing the number of cells entering the S phase of the cell cycle.[13]

Clinical trials have begun testing cetuximab on cervical cancer in human cell and animal models. These studies have demonstrated a decrease in tumor activity as well as an increased production of cluster of differentiation 3 positive thymus cells (CD3 + T cells), CD8 + T cells and NK cells. The increase in the population of immune cells indicates that cetuximab effectively improved immune function and reduces tumor activity.[11,13] These results are promising for the future of cetuximab therapy specifically related to cervical cancer.

Gefitinib

Gefitinib is an orally administered therapeutic drug that prevents phosphorylation and tyrosine kinase activity of the intracellular domain of EGFR. Gefitinib is FDA approved for the treatment of lung cancer. Numerous clinical trials have begun testing the clinical efficacy of gefitinib on various cancers including HPV related cervical cancer.[15,16] Gefitinib is a TKI that targets EGFR. Patients treated with gefitinib have shown signs of improvement and tumor regression in non-cell small lung cancer.[15] Gefitinib binds EGFR and blocks the canonical wingless/integrated Wnt/ß-catenin signaling pathway that is involved in the regulation of cell polarity and migration. The Wnt/ß-catenin signaling pathway plays a key role in the self-renewal of tissues during normal cell turnover. HPV deregulates this activity enhancing the epithelial-mesenchymal transition (EMT) of cancerous cells. The process of EMT allows cancer cells to suppress epithelial features and leads to increased cellular migration or cell renewal.[11] When the Wnt/ß-catenin pathway becomes active due to the presence of HPV, ß-catenin accumulates and accelerates the EMT process. This is a crucial pathway for the survival of HPV related cervical cancer cells.[2,16] By blocking the *Wnt/ß-catenin* pathway gefitinib can stop the EMT process and induce cell apoptosis and cell cycle arrest of cancerous cells.[16] The use of gefitinib for the

treatment of HPV related cervical cancer is in clinical trials. The results have shown gefitinib to be a promising therapeutic option for the future treatment of HPV related cancer.[11]

Discussion

Targeted molecular therapy has shown to be successful compared to traditional cancer therapies. Within the past ten years many molecular targeted therapies have been developed that successfully increase patient survival and decrease the rate of disease recurrence. One example is the drug imatinib. Imatinib is considered one of the more successful molecular targeted therapies developed for treating leukemia and gastrointestinal tumors without the negative side effects of chemotherapy.[12] Targeted therapy focuses on specific genetic alterations in various types of cancers making it more effective, highly specific, and more tolerable than other therapeutic methods such as radiotherapy or chemotherapy. Although chemotherapy is effective, it deals with rapidly dividing cells, destroys both cancerous and normal cells, and can result in long term toxic side effects for the patients making it a less desirable option.[6,15] Molecular targeted therapy is successful independently and in combination with traditional chemotherapy. Studies have shown that combining molecular targeted therapy with chemotherapy can decrease the development of therapeutic resistant cancer cells and successfully reduce tumors when compared with the treatment of cancer patients receiving a single drug therapy.[6] Molecular targeted therapy also reduces the likelihood of disease recurrence. By developing drugs to target the molecular pathways responsible for producing treatment resistant cancer cells, aggressive carcinoma cells can successfully be destroyed and increase a patient's overall survival rate.[7]

Although molecular targeted therapy is promising, it does have limitations. The high specificity of a molecular target limits the broad use of the drug in cancer treatment. Targeted therapy is effective when the drug targeted biomarker is responsible for progression of the patient's cancer.[6] Cancer can be unpredictable. There are numerous causes or genetic mutations that are responsible for the progression of cancer. A drug with one specific molecular target will not work on every cancer and as a result, will not be effective for the treatment of every patient. It is essential to understand the biology behind HPV related cancers when selecting the molecular targets that will be beneficial to the most patients.

Another limitation associated with targeted therapy is the development of drug resistance. Resistance has been identified upon initial cancer diagnosis due to the presence of drug-resistant mutations or a patient may develop a drug resistance during progression of the disease.[17] Mutations present in the PI3K pathway have been associated with the development of ceftuximab resistance during therapy. In HPV patients specifically, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PI3KCA*) mutations cause uncontrollable cell growth and deregulates the PI3K pathway. This specific mutation is associated with poor prognosis and treatment response when compared to patients without the mutation.[5] Clinical trials aimed at preventing cetuximab resistance are utilizing combination treatments with additional molecular targeted therapies such as mesenchymal epithelial transition (MET) and proto-oncogene tyrosine-protein kinase (SRC) inhibitors.[14] Cetuximab resistance has also been linked to the nuclear accumulation of EGFR which increases cellular proliferation through SRC signaling. This results in the patient becoming desensitized to cetuximab therapy. Clinical studies have found that nuclear translocation of EGFR can be prevented by using additional molecular targets such as an SRC family kinase (SFK), non-receptor tyrosine kinases responsible for signaling, to overcome resistance and increase cell death.[11,13]

A common mutation causing resistance towards gefitinib therapy is the Thr790Met (*T790M*) substitution. This mutation replaces a threonine (T) with a methionine (M) at the

entrance of the adenosine triphosphate (ATP) binding site preventing TKIs from binding.[18] Over half of all lung cancer patients treated with gefitinib will develop a *T790M* mutation at the beginning of disease progression.[17] Although this is common in lung cancer cases, that does not necessarily mean that *T790M* mutations will occur in HPV patients. However, since gefitinib is still in the clinical trial phase for HPV, further research will be required to determine if *T790M* will play a significant role in gefitinib resistance in the treatment of HPV patients.

Conclusion

The recent advancements in molecular targeted therapy for HPV related cancers is a promising therapy that can be prescribed individually and in combination with chemotherapy. Disease recurrence remains a cause of HPV related cancer death.[2] By implementing targeted molecular therapy, it is possible to decrease the likelihood of disease recurrence by targeting specific molecular pathways responsible for producing therapy resistant cancer cells.[7] Cetuximab and gefitinib have provided promising evidence for safe and effective treatment options for patients with HPV related cancer.

Cetuximab has proven to be a safe and effective FDA approved molecular targeted treatment option for HPV related HNSCC. It is responsible for an increase in the overall survival rate of patients with both recurrent and locally invasive cancers.[14] Cetuximab has provided additional therapy options to patients and has demonstrated the effective use of molecular targeted therapy in the treatment HPV related cancer.[13] Clinical trials testing gefitinib on cases of advanced HPV related cervical cancer have shown the drug to be safe and effective therapy. The high expression of EGFR in cervical cancer facilitates the binding of gefitinib leading to the destruction of cancerous cells.[11] There is great potential for the use of molecular targeted therapy in the treatment of HPV related cancer as well as other cancers. However, the development of drug resistance remains problematic and indicates a need for the development of additional targeted therapies to be used independently or in combination with existing treatments.

Various studies have shown that combining different drugs as part of a patient's therapy provides an improvement in treatment outcomes and decreases the development of drug resistance. Combining chemotherapy or additional molecular therapy targeted drugs provides a mechanism to potentially combat the development of drug resistance and improve a patient's survival rate.[6] With the limited options available for molecular therapy in HPV cancer, additional research is required to develop efficient targeted therapies.[5] Given that the genetic makeup of each patient's cancer can be different, the development of new molecular targeted therapies has the potential to eliminate invasive and toxic treatments while simultaneously paving the way for advancements in precision medicine.[6]

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Review article

CAR-T Immunotherapy Limitations and Advancements for Relapse and Refractory Pediatric B-Cell Malignancies

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Chimeric antigen receptors (CARs) are genetically engineered, T-lymphocyte receptors that target hematological cancer cells and solid tumors. CAR-T cell immunotherapies for B-Cell malignancies target CD19 positive cells and have shown dramatic results in treating pediatric patients since becoming licensed as tisagenlecleucel by the US Food and Drug Administration in 2018. Tisagenlecleucel is used to treat relapsed or refractory B-cell acute lymphoblastic leukemia (ALL) and large diffuse B-cell lymphomas (DLBCL) in children and young adults up to age 25. While CAR-T immunotherapies have shown continued promise, crucial limitations are being investigated to improve anti-CD19 CAR-T toxicity, off-target events, bridging and dual treatment requirements, and manufacturing capabilities. This review examines the current limitations and advancements of anti-CD19 CAR-T immunotherapy for pediatric B-cell malignancies focusing on relevant improvement strategies for engineering, efficacy, and patient safety.

Key words: Anti-CD19 CAR-T cells; pediatric acute lymphoblastic leukemia; pediatric diffuse large B-cell lymphoma; replapsed/refractory B-cell malignancies; limitations of Anti-CD19CAR-T cell therapy; anti-CD19 CAR-T manufacturing advancements; cytokine release syndrome; neurotoxicity; immunotherapy

Introduction

Chimeric antigen receptor T-cell (CAR-T) immunotherapies have been a revolutionary advancement in treating various cancers, specifically in hematological malignancies. There are currently over 500 active CAR-T clinical trials worldwide and 6 Food and Drug Administration (FDA) approved commercial products since 2017.[1] Anti-CD19 CAR-T cell therapy in the treatment of B-cell malignancies has been significantly successful in providing long-term remission for patients up to age 25.[2] Tisagenlecleucel was the first FDA approved CD19- CAR-T therapy and is the new standard of care for relapsed and pediatric refractory to young adult B-cell acute lymphoblastic leukemia (B-Cell ALL).[3] Since FDA approval in 2018, tisagenlecleucel has treated over 5,400 pediatric patients.[2] As CAR-T approvals expand and production technology advances, increasingly more patients are anticipated to receive targeted immunotherapies.

Prior to CAR-T advancements, patients with B-cell malignancies received multiple lines of intensive myeloablative chemotherapy regimens. While complete remission (CR) is observed in nearly 80% of cases with standard

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treatment, many patients end up with relapsed and refractory disease within 5 years.[4] Intensive chemotherapy requires the destruction of cancer and healthy cells, which is highly taxing on patients. Historically, allogeneic hemopoietic stem cell transplantation (allo-HSCT) was the only curative option to overcome relapse.[5] However, the risk of graft-versus-host disease (GVHD) and a high relapse rate has remained a continued challenge in treating residual disease. Ultimately this standard of care requires continued, life-long treatment.

Within the last 5 years, however, CAR-T immunotherapy has shown promise in the field of oncology with novel technology to target cancer-antigens. Pediatric patients with relapsed and refractory B-cell malignancies who have undergone CD19-targeted CAR-T immunotherapy show 70-90% CR.[6] These results vary between institution due to the dual therapies administered to prevent toxicity and infection following treatment.[6] CR has been most effectively reported in pediatric B-cell ALL compared juvenile cases and diffuse large B-cell lymphoma.[4] Cytotoxic effects, such as cytokine release syndrome (CRS) have been observed in 77% of patients treated with tisagenlecleucel.[7] CRS is graded by severity and can be potentially life-threatening.

While promising, these novel targeted immunotherapies have critical limitations that are currently being investigated. These limitations include severe cytotoxicity such as CRS and neurotoxicity, off-target CAR-T functionality, and manufacturing constraints. This review article discusses the current limitations and advancement strategies for engineering safe and effective CD19 CAR-T immunotherapy for pediatric relapsed and refractory B-cell malignancies.

Background

Pediatric B-cell malignancies include B-cell ALL and DLBCL. B-cell ALL is the result of rapid growing lymphoblasts developing from B-cell precursors which target the immune system.[4] Eighty percent of B-cell ALL patients are infants and juveniles.[4] DLBCL is the most common form of non-Hodgkin lymphoma (NHL) and represents 10-20% of pediatric NHL cases.[4] DLBCL arises from the malignant development of B-cells typically occurring in lymph nodes.[4] In both forms of these B-cell malignancies, the CD19 molecule, which is expressed on all B-cell lymphocytes during each stage of differentiation, acts as a key target for CAR-T targeting agents. The generation of the synthetic T-cell receptors attempt to optimize patient outcomes by enriching naïve, memory, and stem-cell like phenotypes.[8]

CARs are synthetic receptors made with four components including the antigen binding domain, the hinge region, a transmembrane domain, and intracellular signaling domains.[8] The antigen binding domain targets the extracellular cancer surface antigens. Anti-CD19 CAR-T cells are manufactured by isolating a patient's T-cells *ex-vivo* from autologous peripheral blood mononuclear cells (PBMNCs). The CAR gene is inserted via lentiviral transduction, along with the anti-CD19 protein. The cells are then expanded, tested for safety, purity, and potency, and then cryopreserved for later transplantation.[9,10,11] Within a few weeks following transplantation, CAR-T cells will multiply in the body, detect and target CD19+ B-cells for destruction.[3]

Following transplantation, patients are at increased risk of CRS, infection, and neurotoxicity. As CD19 CAR-T cells activate and proliferate, other cell types are activated resulting in high serum concentrations of pro-inflammatory cytokines.[12] Multiple grades of CRS have been reported in 56-100% of pediatric B-cell ALL treatment.[13] Serious adverse reactions can occur within hours of transplantation. Due to these immune reactions, dual treatments are often necessary to limit side-effects, including the use of interleukin-6 (IL-6) corticosteroids.[9] However, due to institution-specific guidelines on treating CRS, present data shows heterogeneous results, thus standard guidelines have not been

established. Current research suggests CAR-T dose compared to bone marrow tumor burden have a positive correlation to CRS severity.[12] Higher doses may not be more effective, as previously thought.[12,13,15,16] However, ongoing research is working to improve CRS symptoms.

Along with the risk of toxicity and infection, the manufacturing process for CD19 CAR-T cells presents limitations. CAR-T cell manufacturing is costly and is limited to sponsor specific manufacturing sites, which significantly reduces patient accessibility. Many patients must travel to prominent cancer facilities to receive treatment. The manufacturing process is complex and requires a pre-determined number of cells for expansion. Pediatric patients with B-cell malignancies already have limited T-cell production, thus manufacturing reproducibility varies by each individual patient. [16,17] The quality in starting material is hard to determine until transduction and expansion occur.[17]

Anti-CD-19 CAR-T cell engineering and manufacturing strategies

Since the advent of CAR-T immunotherapies, there have been four generations of implemented CAR-T technology. The first generation was limited to signaling capacity and inability for sustained cytokine release.[18] The second generation was modified to have additional costimulatory signaling domains which enhanced survival and expansion of activated T-cells. The third generation expanded by having more effective costimulatory domains.[18] The fourth generation has seen further genetic modification to redirect T-cells for universal cytokine-mediated killing (TRUCKs).[18] Finally, the fifth and current generation contains one extra intracellular domain.[19] The evolution of CAR-T generations has focused on improving T-cell activation and costimulatory domains following antigen recognition.[18] While rapid new developments are being investigated, increasing patient numbers versus engineering and manufacturing capabilities poses limitations, specifically for pediatric patient populations.

The starting material for anti-CD19 CAR-T cells is collected via autologous PBMNC apheresis from the patient. For standard hematopoietic stem cell collections, patients receive a mobilization regimen prior to collection to optimize the available stem cells. However, CAR-T cell collections do not require mobilization drugs, therefore the number of CD3+ lymphocytes collected is dependent on the individual patient's disease state and prior chemotherapy bridged with CAR-T therapy.[20] Patient's receiving CAR-T therapy must first undergo chemo-lymphodepletion, which supresses the immune system, allowing for increased CAR-T cell persistence. Most importantly, lymphodepletion requires a favorable microenvironment for CAR-T cells by eliminating homeostatic cytokines.[20]

Genetic factors, medical history, age, and demographics vary from patient to patient; thus, pre-determining treatment efficacy is difficult. Data collected from pediatric T-cell collections shows 77% of patients reaching the collection requirement for starting materials, while 97% of patients only reach the minimum cell count requirement.[20] Factors such as poor collection efficiency or granulocyte and monocyte contamination contribute to these figures.[20] Granulocytes and monocytes pose risk of inhibiting the T-cell enrichment process. The starting leukapheresis material affects downstream anti-CD19 CAR-T production, thus it is critical for the manufacturing facilities to set cell count starting requirements to optimize downstream T-cell isolation processes. However, these collection requirements may be under- or over-estimated between patients, resulting in insufficient collections or excess material which is discarded.

Following cell collection, products are shipped to the manufacturing facility. The cells must maintain stable temperature as deviations will affect the cell's viability. To mitigate facility limitations, cells shipped for tisagenlecleucel manufacturing are cryopreserved and thawed for production, which has been shown to significantly reduce cellular viability.[20] Currently, there is only one facility

in the United States that manufactures tisagenlecleucel, located in Morris Plains, New Jersey, but other international facilities are being developed.[17] CAR-T cells are considered phase 1 investigational drugs; therefore, the FDA sets regulations for good manufacturing practices (GMP).[21] These regulations include guidance for personnel, quality control (QC), facilities and equipment, manufacturing and record keeping, laboratory controls, packaging, labeling, distribution, as well as cell and gene specific requirements.[21] These regulations ensure purity, potency, and safety of the final product. Due to the stringent nature of FDA and other regulatory adherence, expanding facilities is a complex, costly process.

The manufacturing process requires the use of unidirectional and multidirectional flow of materials and staff to limit risk of contamination or cross-contamination.[17] Advanced instrumentation is required for each facility manufacturing anti-CD19 CAR-T cells. The starting material is first washed and fractionated; CD3+ T-cells are selected using an antibody-conjugated magnetic bead process, followed by T-cell activation with the 4-1BB costimulatory domain and anti-CD19 CAR complimentary DNA (cDNA) transduction with retroviral vectors and mRNA electroporation.[22] The domain, 4-1BB, is expressed on activated T-cells and natural killer (NK) cells and acts as an inducible costimulatory receptor. In the malignant B-cell environment, 4-1BB restores effector functions of dysfunctional T-cells, therefore its use in CAR-T manufacturing has been promising.[22]

Antigen-presenting cells, such as dendritic cells and artificial antigen-presenting cells, are used to stimulate the expansion of CAR-T cells.[17] *Ex vivo* expansion of T-cells is only accomplished with sustained activation.[22] This step is the area of research focused on producing more effective CAR-T generations. Following activation and expansion, the anti-CD19 CAR-T cells are then tested and ensured for purity, potency, and safety prior to shipment back to the treating facility.

Critical quality control points are essential for maintaining and producing quality product. It is important that the starting material is properly enriched to eliminate unwanted cells for selection. CAR expression indicates efficacy of stable expression post-transplantation. The ratio of CD4+ and CD8+ T-cells must be maintained to prevent downstream CRS toxicity.[23] Each step in the engineering and manufacturing process must be evaluated with checkpoints.

Cytokine release syndrome and neurotoxicity

CRS and neurotoxicity are noted as the most prominent and severe reactions following anti-CD19 CAR-T immunotherapy, caused by generalized immune system activation. The introduction of anti-CD19 CAR-T cells results in immune cell cross activation and systemic cytokine release.[24] Severe symptoms of CRS include, fever, rigors, hypotension, kidney failure, coagulation, hypoxia, and respiratory distress.[25] Neurological toxicities are observed as a separate symptom; however, cytokines have been implicated in causing neurotoxic symptoms.[25] Neurotoxic symptoms include headache, encephalopathy, delirium, tremors, peripheral neuropathy, seizures, and aphasia.[26]

The onset of CRS is observed by elevated levels of inflammatory cytokines such as interferon-gamma (IFN- γ), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), and interleukin-12 (IL-12).[26] CRS is graded by severity on a scale of 1 to 4. CRS has historically been graded with variability among institutions, thus the data has been difficult to compare due to this unclear consensus. Various grading systems have been found to have overlapping symptomatology and described using differing terms.

In 2018, the American Society for Transplantation and Cellular Therapy (ASTCT) developed consensus recommendations to establish a grading system based on symptomatic algorithms.[25] The current, established grading system determined by ASTCT is as follows: CRS

grade 1 is noted as a mild reaction such as fever; grade 2 is noted with hypotension and mild hypoxia which is quickly resolved with non-steroid anti-inflammatory drugs (NSAIDs), and prophylactic medications; grade 3 is indicative of hypotension requiring vasopressors or clinical sequelae requiring prolonged treatments; and grade 4 indicates life-threatening symptoms requiring urgent intervention, organ failure, or ventilatory support.[25]

CRS symptoms are observed within the first 14 days following immunotherapy initiation. Data from patients treated with tisagenlecleucel shows 77% of patients experience some grade of CRS within the first 3 days of treatment, and 48% of patients experience CRS graded ≥ 3 within the first 8 days of treatment.[27] Due to this, patients are closely monitored for 4 weeks following CAR-T transplantation. Tocilizumab, which blocks the inflammatory IL-6 protein, is the gold standard for treatment of CRS. [26,28] Tocilizumab is a human IL-6 receptor monoclonal antibody originally approved to treat rheumatologic diseases. In 2017, the drug was approved by the FDA to treat CRS in patients 2 years of age and older.[29]

Tocilizumab has been shown to be effective in treating COVID-19 symptoms, which has resulted in severe shortages of the drug.[30] To mitigate these unexpected drug shortages, current studies are exploring the use of anakinra, a recombinant IL-1 receptor antagonist, which is also an FDA approved drug used for rheumatologic disease in treating pediatric CRS and immune effector cell-associated neurotoxicity syndrome (ICANS).[31]

ICANS represents a more diverse symptomatic profile than CRS. Early signs of ICANS are indicated by expressive speech patterns, tremors, attention impairment and lethargy.[25] Most notably, pediatric patients with symptoms of expressive aphasia have shown to be a hallmark of ICANS diagnoses.[25] Like CRS grading, ICANS is graded on a severity scale from 1 to 4. Neurological and encephalopathy assessments are evaluated twice daily to monitor neurotoxicological events following anti-CD19 CAR-T transplantation. Pediatric ICANS is reported in conjunction with CRS symptoms, at a median of 2 days following CRS onset.[32] While ICANS symptoms are generally reversible, 47% of pediatric patients impacted with ICANS following anti-CD19 CAR-T therapy required admission to the intensive care unit (ICU).[33] Recovery from severe ICANS ranges from 27-75 days.[34] Risk of developing ICANS has been correlated with early CAR-T expansion *in vivo,* however more research is needed.[35]

Anti-CD19 CAR-T cell efficiacy

Despite the success of anti-CD19 CAR-T immunotherapy for pediatric patients, poor clearance of malignant cells and prominent adverse events complicate the therapy's efficacy and safety. There are currently approximately 200 clinical trials studying pediatric anti-CD19 CAR-T immunotherapies.[35] Many of these studies focus on improving the pharmacokinetics and safety of anti-CD19 immunotherapies and optimizing chemotherapy and bridging therapies.

The efficacy of 5th generation anti-CD19 CAR-T therapy requires pre-treatment chemotherapy and bridging therapy, however there are few patients who receive treatment without bridging. Bridging therapy, which is determined by the treating facility, greatly increases the efficacy of anti-CD19 CAR-T activity. Bridging therapy involves high-dose chemotherapy, followed by low dose chemotherapy, radiation therapy and anti-CD19 CAR-T-transplantation coupled with symptomatic treating agents.[15] As previously discussed, this process eliminates circulating lymphocytes and cellular contaminants and reduces the risk of disease progression prior to transplantation. Due to the complexity of bridging therapy, current studies aim to optimize treatment approaches, as continued chemotherapy and radiation can be taxing on the patient. The lymphodepleting bridging therapy occurs for 4 weeks prior to transplantation.[36] Diagnostic analysis of minimal residual disease (MRD) is

currently not a required measurement prior to T-cell collection or CAR-T manufacturing; however, evidence supports MRD measurements correlate with the probability of CAR-T efficacy.[36]

Since original licensing, the five-year tisagenlecleucel treatment data shows a 55% survival rate of 79 pediatric and young adult B-cell ALL patients.[27] Relapse-free survival data indicates that 44% of patients remained in remission for a median of 43 months.[27] Prior to anti-CD19 CAR-T immunotherapy, the five-year survival rate for pediatric and young adult patients was 10%.[27] Reported efficacy analysis was based on determination of the rate and duration of response. Dual treatment tocilizumab indicates 298% higher area under the curve (AUC) from day 0 to day 28 compared to patients who only received corticosteroids. [27] Prior HSCT was noted in 47% of treated pediatric patients.[27]

CAR engineering is specifically important in understanding efficacy. As anti-CD19 CAR-T cell generation evolves, strategies to engineer enhanced antigen recognition with minimal immune activation remain the focus. Current research is evaluating the efficacy of bispecific ligand-binding domains in which two antigens activate the specific CAR.[37] This design shows promising evidence of eliminating antigen escape *in vivo*. However, off-target toxicity is still observed. Alternatively, molecular CAR expression and stability has been shown to correlate with alteration of the hinge and transmembrane domains.[37] Manipulation of the length of the hinge and transmembrane domains not only correlate with potency, but also significantly reduces neurotoxicity as observed in a phase I clinical trial.[38]

Conclusion

New strategies for anti-CD19 CAR-T development are rapidly expanding, as researchers and clinicians gain new insights on the efficacy and safety of design and administration. CAR-T cell therapy has significantly increased survival rates in relapsed and refractory pediatric B-cell malignancy as compared to conventional chemotherapy interventions.[4,8,27] Anti-CD19 CAR-T immunotherapy has greatly improved over the span of 10 years in clinical trials and the 5 years since FDA approval. Targeting more specific molecular and cellular pathways while limiting off-target toxicity remains the most prominent improvement strategy. As anti-CD19 CAR engineering improves, manufacturing efforts must also expand to extend patient access. Examining MRD diagnostic data to develop patient specific CARs has shown promise in the development of more individuals CAR-T immunotherapies.[36]

Future pediatric treatment strategies aim to develop anti-CD19 CAR-T cells from healthy donors to eliminate risk of disease burden cells affecting efficacy.[39] Patients prior treatments limit the effectiveness of the CAR-T cell product due to T-cell dysfunction. Allogeneic anti-CD19 CAR-T cell therapy has been shown to be effective in adult DLBCL, Chronic Lymphocytic Leukemia (CLL), and Mantle Cell Lymphoma (MCL).[40] Developing allo-CAR-T cells come with its own set of complications, including donor recruitment strategies, human leukocyte antigen (HLA) matching, and limiting the risk for GVHD in the recipient.

Ultimately, current anti-CD19 CAR-T cell immunotherapies has greatly reduced relapse and refractory disease in pediatric B-cell cancers. As the understanding of malignant-burdened molecular pathways expands, improved generations of CARs will advance. Future directions aim to suppress cytotoxic effects and reduce required bridging therapies and dual treatments.

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Review article

Human Blood Cell Pathophysiology Associated with Acute COVID-19 Infection

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December 2019 marked the beginning of an outbreak of coronavirus disease (COVID-19 or SARS-CoV-2), which occurred in Wuhan City, Hubei Province, China. The most publicized effect of this virus is its impact on the human lungs, damaging the walls and lining of the air sacs causing respiratory symptoms, such as difficulty breathing and chest pain. This literature review highlights the changes in erythrocytes, leukocytes, and platelets during COVID-19 infections. Publicly available articles and reports related to COVID-19 infections on blood cell populations were collected and summarized. COVID-19 viral infections alter erythrocytic glycolytic pathways and membranes, and the ability to transport and deliver oxygen. T-cell lymphopenia is common among COVID-19 patients. The leukocytes produce excess inflammatory products during a "cytokine storm" where T and B lymphocytes, natural killer cells, neutrophils, and macrophages secrete pro-inflammatory cytokines to attenuate natural killer function to resolve inflammation. This further activates cytokine release from neutrophils and macrophages which, in turn, leads to exponential increase in inflammation, release of reactive oxygen species, superoxide anion, and nitric oxide, and associated tissue damage. With respect to platelets, thrombocytopenia has been observed in COVID-19 patients. The virus infects bone marrow cells resulting in abnormal hematopoiesis, direct destruction of platelets associated with the cytokine storm and immune function, and increased platelet consumption and tissue damage caused by the virus. The function of blood cell populations is severely compromised by SARS-CoV-2, contributing to severe disease outcomes, such as tissue and organ damage, and death. As more information is available about the virus, vaccines and the effect on erythrocyte, leukocyte, and platelet pathophysiology, this information will be used to develop new therapeutics to improve the recovery of patients from this disease.

Key words: COVID-19, SARS-CoV-2, erythrocytes, leukocytes, platelets

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the third virus to have caused coronavirus-related outbreaks in humans over the past 20 years; following the severe acute respiratory syndrome (SARS) from 2002–2004 and in 2012 the Middle East respiratory syndrome (MERS).[1] Through comparing the different coronavirus species, it has been identified that SARS-CoV-2 is far more infectious, the overall number of deaths has far

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exceeded fatalities from SARS and MERS.[2] SARS-CoV-2 has caused a global pandemic of acute respiratory disease, namely the coronavirus disease 2019 (COVID-19), and its high transmissibility has made it a significant international health concern. Most of those infected will experience mild to moderate respiratory symptoms and will recover without special treatment. However, those with an underlying health condition (i.e., immuno-compromised) or the elderly, are more likely to become severely ill and may die from COVID-19. Many published studies and literature reviews have highlighted the respiratory symptoms associated with SARS-CoV-2 infection, while media coverage of the vaccines used to prevent COVID-19 infections has focused on the thrombotic issues caused by the vaccines. This literature review aims to explore and summarize the effects that the SARS-CoV-2 virus has on human erythrocytes, leukocytes, and platelet populations; and how their function is compromised by the SARS-CoV-2 virus and contributes to the disease symptoms and organ damage.

Effect of COVID-19 infections on erythrocytes

Erythrocytes, also known as red blood cells (RBCs), are the most common type of cell found in human blood, accounting for approximately 40% of the total blood volume. The main roles of RBCs are to transport gases including oxygen and carbon dioxide between the lungs and tissues. Therefore, disorders in morpho-physiology or function may result in tissue hypoxia and damage. In a recent review, researchers indicated that patients with COVID-19 infection present with abnormal red cell morphology with RBCs exhibiting the presence of anisocytosis, spherocytosis, stomatocytes, and polychromasia.[3] It has been demonstrated that hematological and morphological changes occur impairing RBC deformability post recovery from mild COVID-19 disease, stiffening the RBCs.[4] These changes coupled with alterations in hemoglobin, may also be associated with hypoxemia during COVID-19 infection. While Nader observed RBC aggregation as another crucial rheological parameter that is affected by acute severe COVID-19 disease.[5]

SARS-CoV-2 also affects RBC function by causing damage to the membrane proteins, which indirectly regulates their capacity to release oxygen and allows them to squeeze through capillaries. COVID-19 infections have an impact on the oxidation process of band 3 and binding with spike 1 protein (S1).[6] Alterations to the band 3 anion transport protein can result in changes to the ATP release mechanisms of RBCs, and thus, reduces vasodilation and oxygen delivery to tissues, causing hypoxia in patients. To further support this notion, analysis of the RBCs from COVID-19 patients was completed using real-time deformability cytometry. An anomaly in structure was observed, which was mainly characterized by the appearance of small RBCs with low deformation in typical channel flow conditions.[7] More specifically, the median deformation of the RBCs exhibited a weak decrease in COVID-19 patients compared with healthy donors and recovered patients. In addition, lipidemic analyses also observed that the membrane of the RBCs from infected persons had lower levels of short-chain fatty acids and increased long-chain saturated fatty acids.[8] Furthermore, as mature RBCs cannot synthesize new proteins to replace damaged ones, and the average lifespan of an RBC is 120 days, it was hypothesized that the circulation of irreversibly damaged RBCs with impaired functions contribute to the long-term effects of COVID-19.[8]

Researchers have also reported that the RBCs in those infected by the SARS-COV-2 virus showed significant alterations in glycolysis and more specifically exhibited elevated sucrose consumption.[8] This change in the glycolytic pathway in the RBCs of COVID-19 patients is likely due to infected individuals having higher levels of phosphofructokinase, an enzyme that

limits the rate of glycolysis. Another possibility is that SARS-CoV-2 in the human cell activates mitochondrial oxidative damage, which upregulates intracellular production of reactive oxygen species, which then increases cellular injury, intracellular stress, and increases the concentration of glucose in the infected cell. Consequently, cellular glucose metabolism becomes altered, changing the final product of the glycolytic pathway.[8] RBCs of COVID-19 patients also exhibit increased levels of glycolytic metabolites.[8] COVID-19 infection induced hypoxia affects 20% to 40% of patients and it was theorized that the increase in glycolytic metabolites was to counteract these effects. Glycolytic metabolites enhance the capacity of hemoglobin to off-load oxygen, thus providing sufficient oxygen to the tissues. However, this limited RBC capacity to readily respond to environmental variations in hemoglobin oxygen saturation causes some of the respiratory symptoms experienced by those with COVID-19.

Changes in leukocyte function with COVID-19 infections.

Leukocytes account for approximately 1% of a human's total blood volume and normal concentrations in human blood varies between 4,000 and 10,000 cells per microliter. Macrophages typically arise from bone marrow precursor cells, which develop into monocytes, and function to phagocytose pathogens, release pro-inflammatory cytokines, and antimicrobial mediators. Neutrophils are similarly produced in the bone marrow and rapidly respond to trap and remove invading pathogens. T cells mature in the thymus and mainly function to kill infected host cells, produce cytokines, and regulate the immune response.[9] T cell lymphopenia occurs when there is an abnormally low number of T lymphocytes in the blood. It is currently unknown as to why COVID-19 infection causes a decrease in T lymphocyte levels, but it has been suggested that the inflammatory cytokine storm is likely a key factor behind the observed lymphopenia.[10]

Previous studies regarding COVID-19 infection and the human immune system have noted that infection with SARS-COV-2 is accompanied by an aggressive inflammatory response in an event known as the "cytokine storm."[11] This involves the release of a large number of pro-inflammatory cytokines by cells including T and B lymphocytes, natural killer cells, neutrophils, and macrophages.[11,12] The cytolytic function of natural killer cells is diminished in some forms of the cytokine storm. This can lead to prolonged antigenic stimulation and difficulty resolving inflammation. Neutrophils produce an extracellular network of fibers that amplify cytokine production during the cytokine storm and macrophages can become activated and secrete excessive levels of cytokines, ultimately causing organ failure.[12]

Another consequence of the "cytokine storm" is the overproduction of pro-inflammatory cytokines, which is due to a loss of negative feedback in the immune system.[13] The main cytokines involved in the induced cytokine storm are interleukins 1 and 6, tumor necrosis factor alpha, colony stimulating factors, growth factors, and interferons. These cytokines exert positive feedback on other immune cells, including macrophages, neutrophils, and T cells; which are recruited to the site of infection in excessive levels, leading to the exponential increase of inflammation.[13] The increase in these three types of immune cells and the resulting exacerbation of the inflammatory process can produce destructive results on human tissue, which leads to the destabilization of endothelial cell to cell interactions, vascular barrier, and capillary damage.[11] In addition, increased cytokine production the release of reactive oxygen species, superoxide anion, and endogenous nitric oxide are induced; which all contribute to the myocardial damage experienced by some COVID-19 patients.[14] The cytokine storm is also associated with a downregulation of angiotensin-converting enzyme 2 receptor (ACE2), which leads to an increase in angiotensin II and stimulation of angiotensin II

receptor type 1 (AT1R). This is believed to be the causative factor for severe lung complications in several COVID-19 cases. However, at this stage, the driver of the cytokine storm in COVID-19 patients is unknown.[12]

Effect of COVID-19 infections on platelet function

Human blood contains 150,000 to 400,000 platelets per cubic millimeter. Thrombocytopenia is a condition affecting those with an abnormally low platelet count. A study involving 1,099 patients from 31 provinces/direct-controlled municipalities in China showed that 36.2% had thrombocytopenia, suggesting the possibility that COVID-19 infections interfere with the platelet count.[15] The possible mechanism of thrombocytopenia in COVID-19 patients involves multiple theories. First, SARS-CoV-2 infections may directly reduce platelet production causing thrombocytopenia. Coronaviruses can infect bone marrow cells and cause abnormal hematopoiesis through receptors such as the aminopeptidase N (CD13) receptor, which decreases the production of platelets. This receptor removes the N-terminal amino acids from unsubstituted oligosaccharides, allowing it to regulate the activity of hormones, cytokines, and chemokines; which take part in inflammation.[16] This theory is further supported by others who speculated that after being impacted by the cytokine storm induced by COVID-19 infection, the hematopoietic progenitor cells in bone marrow are destroyed, thus decreasing platelet production.[17] The second possible reason as to why thrombocytopenia occurs is due to the possibility of COVID-19 infection increasing platelet destruction.[15] COVID-19 infection increases the levels of autoantibodies and immune complexes, resulting in destruction of platelets by the immune system. This is due to the production of antibodies which bind to antigens on platelets through molecular mimicry. The third possible hypothesis is that COVID-19 infections may increase platelet consumption, as being infected leads to damaged lung tissues and pulmonary endothelial cells, and hence, there is an increased utilization of platelets.[18]

COVID-19 infections have also been found to alter gene expression in platelets, as well as platelet-specific granule content. Two-related genes, S1000A8 and S1000A9 are upregulated in the platelets of COVID-19 patients, resulting in the increased production and release of myeloid-related proteins 8 and 14, which activate endothelial cells to promote an inflammatory hypercoagulable state. Platelet membranes contain $P2Y_{12}$, a Gi-coupled receptor expressed on platelets, which regulates ADP-induced platelet aggregation. At the same time $P2Y_{12}$ receptor activation contributes to the inflammatory process. This suggests that targeting the adenosine diphosphate receptors $P2Y_{12}$ on the platelets in COVID-19 patients may reduce proinflammatory platelet-endothelial interactions.[19] The myeloid-related proteins activate endothelial cells, promoting an inflammatory hypercoagulable phenotype, which leads to higher levels of clotting and inflammation in vessels, and hence, greater disease severity.

Researchers have also determined that platelet-specific granule content levels are elevated in patients and linked to increased platelet activation, resulting in platelet hyperactivity in severe COVID-19 cases. Other gene-expression changes that occur in platelets from COVID-19 patients involve pathways associated with protein ubiquitination, antigen presentation, and mitochondrial dysfunction, which increases platelet activation and aggregation that was caused by increased mitogen-activated protein kinase pathway activation and thromboxane generation. As such, COVID-19 infection is associated with platelet hyperreactivity, which may contribute to COVID-19 pathophysiology.[20]

Conclusion

The current COVID-19 pandemic is a highly contagious pathogenic viral infection which has become an international public health problem. Infected persons may be asymptomatic or suffer from mild to severe symptoms,

including fever, coughing, and diarrhea. There have been rapid advances in the world's understanding of the SARS-CoV-2 pathogen, clinical characteristics of COVID-19 infection, and development of COVID-19 vaccines. This literature review highlights the impact of SARS-CoV-2 infections on blood cell populations and how they can contribute to the pathophysiology of the disease.

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Research article

Whole Blood Viscosity: Affordances and Re-evaluation of Sensitivity and Specificity for Clinical Use

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Over the years, whole blood viscosity (WBV), an indicator of thickness and stickiness of blood has been a laboratory marker for blood stasis, and useful for monitoring several disorders including cardiovascular diseases (CVD). However, the use of WBV in clinical practice is still limited by affordances, knowledge and attitude. With the development of extrapolated whole blood viscosity (eWBV) method from haematocrit and total serum protein level, what is yet to be established is the sensitivity and specificity of eWBV to address the limitations in clinical practice. The objective of this study was to highlight the discourse on sensitivity, specificity and affordances (accessibility and affordability) of eWBV to re-evaluate the utilization of WBV in clinical practice, especially in low-mid income communities. This was a observational study that used archived data from haematology and biochemistry routine laboratory tests associated with cardiovascular phenomena. Statistical analysis adopted the conventional paired-contingency table method for sensitivities and specificities to assess validity of eWBV in CVD. Reliability was affirmed by consistent significant differences in WBV levels between thrombocytopenia and thrombocytosis ($p < 0.005$). Calculated validities show that eWBV is ≥64% specific and ≤38% sensitive to cardiovascular phenomena. In conclusion, eWBV is generally less sensitive but more specific for CVD. One major significant finding from this study is that in patients with haematocrit and serum protein results, the risk of bleeding and monitor the effects of therapy can be assessed using specific and accessible eWBV at no extra cost in laboratory service. Being accessible at no extra cost translates to widespread affordance for this laboratory test.

Key words: aspirin, blood stasis, cardiovascular phenomena, clinical practice, laboratory medicine, therapeutic monitoring

Introduction

Monitoring of certain cardiovascular therapies such as antiplatelet therapy includes assessment of resistance, responsiveness and risk of side-effects using routine clinical laboratory methods. Antiplatelet therapies are commonly used in management of chronic diseases including prevention of stroke. According to the American College of Chest Physicians as well as American Heart and Stroke Associations,[1,2] guidelines recommend that in cases of atherothrombosis, stroke or transient ischemic attack, the patient can receive anti-

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platelet agents to prevent recurrence if there are no contraindications.[1-6]

In some patients, the desired effects are not attained when administering aspirin while in others there is the misnomer of aspirin resistance,[7] which can be confused with aspirin non-responsiveness. Furthermore, aspirin may be contraindicated in some patients while indicated in others.[8] Non-responsiveness and resistance to a drug, such as antiplatelet agents, are different phenomena,[9-11] and indeed, there is need for clarification on the definition of antiplatelet resistance in the context of a laboratory 'test' response to a drug.[12] This is regarding the expectation of therapeutic effect i.e. *blood thinning*,[13] which can be determined by a reduction in a patient's 'blood stasis' *vis-à-vis* whole blood viscosity (WBV).[14, 15]

At this juncture, it is pertinent to delineate that WBV is not normally assessed as part of chronic disease management, except at some reference laboratories and there is now extrapolated WBV (eWBV) method to enable testing in routine laboratories. Aggregometry for antiplatelet drug monitoring,[16, 17] is not commonly available outside reference laboratories and rarely accessible in regional health services. Thus, in terms of affordances (accessibility and affordability), there are patients who can afford the cost, but cannot access the service. With advancement in research, eWBV can now be determined from haematocrit and proteinaemia, thus removing the barrier of affordances and making the pathology test available in a clinical laboratory that performs routine haematology and liver function tests.

It is noteworthy that blood viscosity is a cardiovascular phenomenon, which may be associated with other phenomena. For instance, inflammation, metabolic syndrome, and platelet functions are three notable cardiovascular phenomena. Hence, it is hypothetical that laboratory tests for these three phenomena may validate eWBV.

There has been a clarion call for the development of reproducible, simple and user-friendly bedside methods to determine antiplatelet responsiveness;[18] and assessment for bleeding risk is necessary before starting antiplatelet therapy.[19] Therefore, the relevance of this update is in the advancement of a method applicable and commonly available including in low-mid income countries or communities, but also potentially at patient' bedside for diagnostic decisions.

Objective: In the context of knowledge, attitude and, practice (KAP) gap, there is a void that can be viewed as:

- ***What is known***: WBV is a laboratory marker for blood stasis.[9-15]
- ***KAP gap***: WBV is speculated as being too sensitive and less specific for identifying blood stasis.[20]
- ***What is unknown***: sensitivity versus specificity of eWBV to specific CVD phenomena
- ***Proposition***: eWBV is neither too sensitive, nor a less routine CVD marker.

Materials and Methods

Study design and ethical clearance

This study followed a clinical laboratory observational method [21]. It is a clinical laboratory method based on evaluation of archived clinical pathology data; and observational study because it did not utilise experimental intervention but used archived laboratory results. The study was approved by the Human Research Ethics Committee of the University (H2014158).

Setting

Two datasets from electronic laboratory records of two health facilities were used. First dataset was obtained from Australian based South-West Pathology Service (SWPS) of the NSW Health Australia (Fig 1). Second dataset were collected from Wellness House Orange, a General Practice in regional NSW Australia.

Data

Information collected included routine laboratory test indices for assessing WBV and tests for inflammation, metabolic syndrome, and platelet function as three cardiovascular phenomena, which were previously assessed for association.[22-24]

- **Factors of WBV** – haematocrit and serum protein level
- **Inflammation** – white blood cell count (WBCC), erythrocyte sedimentation rate (ESR) and c-reactive protein (CRP). These laboratory test values increase with inflammation.
- **Metabolic syndrome** – blood glucose level, lipid profile. Abnormal values are associated with cardiometabolic syndrome and constitute risk for cardiovascular complications.
- **Platelet function** – platelets count. This test is currently the available closest indicator of blood stasis as a cardiovascular phenomenon.

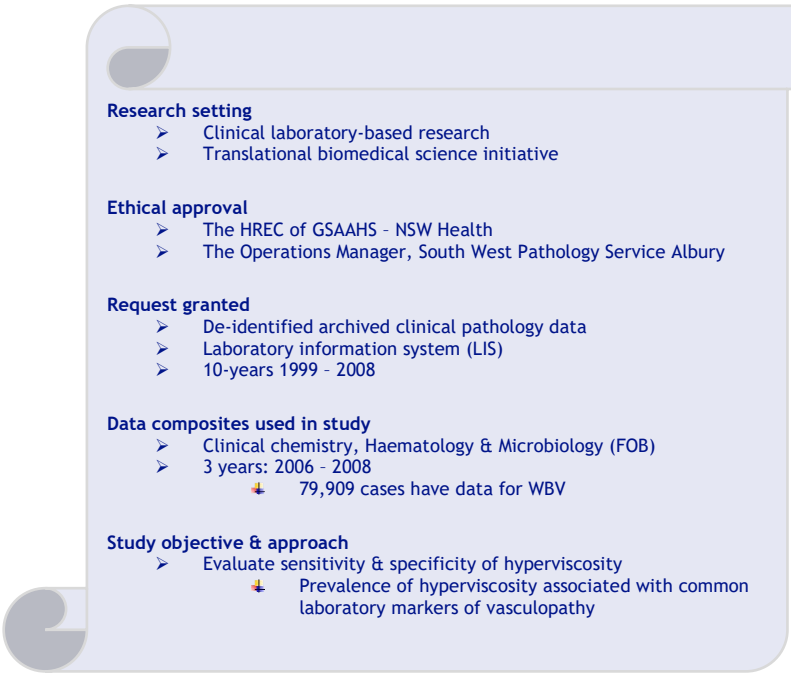


Figure 1: Summary of methods of the 10 years' dataset

Calculation method

First, the eWBV formula was used to generate the blood viscosity levels,[25] and for quick reference, WBV was derived digitally from haematocrit (HCT) and total serum protein level (TP). The translational procedure was as follows:

WBV = (0.17 x (P – 2.07)) + (0.12 x H)

- On excel, it is: eWBV = 0.17*(P–2.07) + (0.12*H)
- P = serum protein level
- H = haematocrit level
- * is multiplication a symbol in excel

Second, WBV sensitivity and specificity were evaluated with reference to determine validity, and this followed the 2-by-2 formula applicable for day-to-day clinical practice.[26, 27]

Statistics

In this study, reliability was defined as consistency,[28] and evaluation was second dataset if results would be consistent with previous observations in the first dataset. Validity meant sensitivity and specificity for the usefulness i.e., utility. Hence, the statistical analysis included to ascertain consistency as well as sensitivity and specificity of eWBV for the three indicated CVD phenomena i.e. inflammation, metabolic syndrome, and platelet function.

Consistency evaluation was done by assessing changes in eWBV levels with the inflammation, metabolic syndrome, and platelet function variables. The paired-contingency table method for sensitivities and specificities was adopted for validity determinations. The 1st and 4th quartiles were adopted as low and high values, respectively, in calculating sensitivities and specificities of the eWBV to inflammation, metabolic syndrome, and platelet function test parameters.

Results

The descriptive statistics of dataset 2 are shown in Table 1. The consistency assessment i.e. *reliability* results are shown in Fig 2, and eWBV is marginally associated with lipidaemia and WBCC i.e. similar to previous report.[24]

Table 1: Descriptive statistics of new dataset

	HbA1c (%)	*WBC×10⁹/L*	*Platelets ×10⁹/L*	*eWBV (208/Sec)*	*TG (mg/dl)*	*CHOL (mg/dl)*
Mean	7.976	7.366	252.351	11.808	118.449	103.090
Median	7.500	7.100	247.000	11.771	102.000	98.500
Mode	7.600	5.800	244.000	11.941	91.000	72.000
SD	2.938	2.242	72.730	0.723	42.827	27.605
Min	4.80	2.60	60.00	9.73	72.00	58.00
Max	44.00	18.10	509.00	14.49	300.00	170.00
N	245	245	245	245	100	100

TG: triglyceride; CHOL: total cholesterol

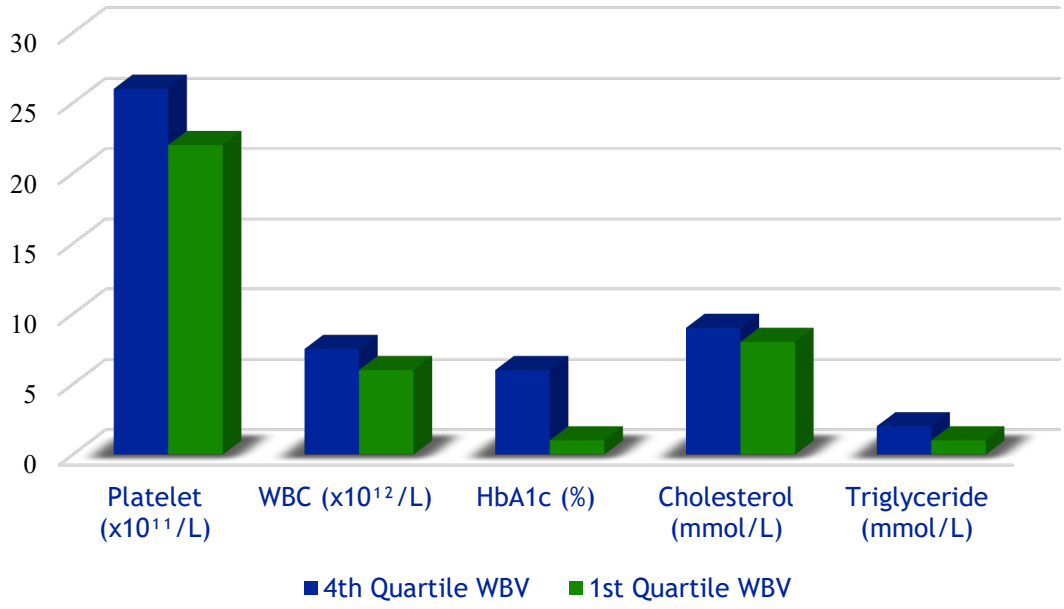
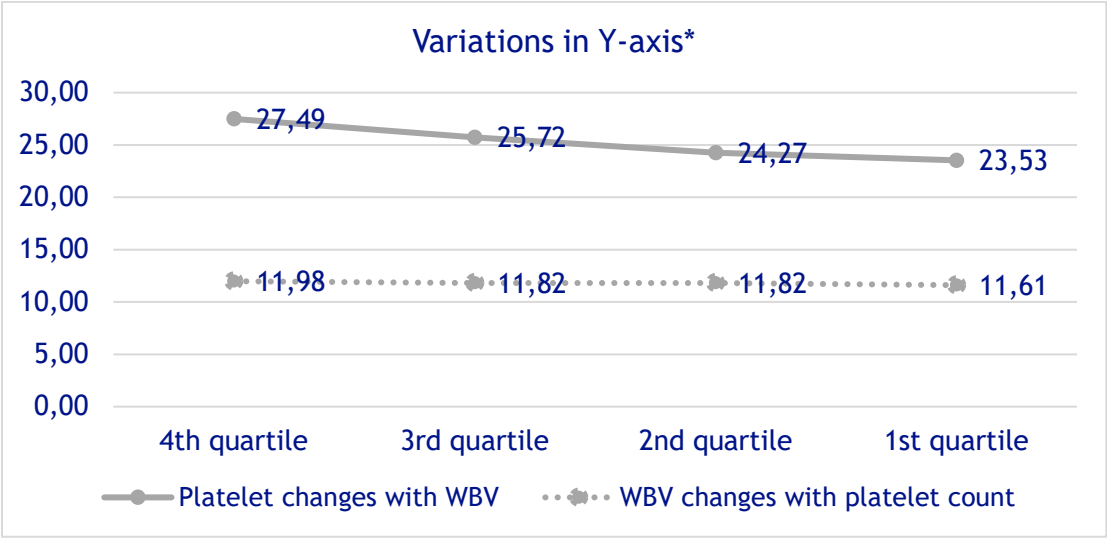


Figure 2: Differences in common clinical variables between quartile groups of WBV



**Platelets ×10⁹/L; and eWBV (208/Sec)*

Figure 3: Reliability of WBV changes with platelet count[29]

Consistent with previous report that platelets count reduced as blood viscosity decreased ($p < 0.001$) and eWBV level signify-cantly lower ($p < 0.005$) in thrombocytopenia compared with thrombocytosis,[22] results from the private general practice showed consis-tency that WBV increased with platelet count and vice versa (Figure 3).

Validity results show sensitivity and specificity of WBV for leukocytosis to be 15% and 69%, respectively. Higher specificity of 78% hypoviscosity to leukocytopenia was observed. Lipidaemia in the dataset were 62% and 38%, respectively for specificity and sensitivity but individual parameters are more specific and less sensitive. On platelet counts, the calcu-lated sensitivity and specificity of WBV for both sides of abnormal platelets showed higher specificity than sensitivity (Table 2).

Discussion

Reliability

Internal consistency assessed the changes in eWBV levels with multiple variables. Based on dataset from private general practice, results affirm consistencies reported[22-24] – that is, WBV may increase with cholesterolaemia, glycosy-lated haemoglobin, leucocytosis, and thrombo-cytosis (Figure 2). Hence it is pertinent to reiterate some points advanced in previous reports that eWBV increased with the level of inflammation, but this was not statistically significant between sub-populations of nega-tive versus positive CRP/ESR results.[30] When eWBV was compared among cases with leuco-cytosis and/or leucopenia, non-significant as-sociation between leucocytosis and hyper-viscosity was observed and this was supported by the low correlation.[24] With reference to platelets as index of platelet function test, *reliability* evaluation showed that as WBV increases with platelet count it is significantly lowered in thrombocytopenia compared with thrombocytosis.[22]

Validity

Validity is sensitivity and specificity,[26] which refers to usefulness and utility. The crux of this discourse is considering leukapheresis to be therapy in leukocytosis-based hyperviscosity and platelet count as an accessible platelets function test. Hence, the extent that high and low WBV are sensitive and/or specific to paradigms of the cell counts was determined and the results show WBV to be more specific and less sensitive to these phenomena (Table 2). Putting the results into perspective, platelets count does not fully reflect the level of blood stasis and this is supported by reports of an association between platelet hyper-activity and antiplatelet therapy.[31]

Gender-specific differences in platelet func-tion and response to antiplatelet therapy are speculated, hence the quest for more specific evidence base.[32] While laboratory monitoring of antiplatelet medication helps to predict individual responsiveness,[33] the significance of this paper is in advancement of sensitivity and

Table 2: Calculated sensitivities and specificities of eWBV to established tests

Established laboratory tests of cardiovascular phenomena		Sensitivity	Specificity
Platelet (150-450 ×10⁹/L)	Thrombocytosis	15%	70%
	Thrombocytopenia	10%	64%
WBC (4.5-11 × 10⁹/L)	Leucocytosis	15%	69%
	Leukopenia	38%	78%
Lipidaemia	Triglyceride > 2.28 mmol/L	17%	97%
	Total cholesterol >5.17 mmol/L	8%	98%
Glycaemic control	HbA1c >6.5%	3%	98%

specificity of an often overlooked test.

Further, significance of this discourse is on accessibility of eWBV, bearing in mind that blood viscosity test is not readily available or accessible but eWBV can be extrapolated from haematocrit and serum protein levels,[25, 34] just as INR is generated for prothrombin time. Therefore, affordances may not be an issue in providing blood viscosity test but what is needed is awareness.

Note on study limitation:

Several studies have reported inconsistent results possibly due to the objectives and protocols. For instance, cognizance was taken that serum protein level excludes fibrinogen, but this was deemed not a major issue since the estimated formula is more specific and less sensitive. Correlation of mean arterial pressure with WBV has been reported to be different,[35, 36] but differences in genders' haematocrit were noted as not being compensated in one.[35] Further, this study focused on platelets and associated routine laboratory tests with recourse to 'blood thinning' effect of antiplatelet therapy. Notwithstanding this limited focus, the validity of assessing level of blood stasis for antiplatelet contraindication in thrombocytopaenia or indication in thrombocytosis is presented to advocate evidence-based prescription. It has been reported that WBV strongly correlates with blood salicylate level and international normalized ratio;[22, 37] therefore, this report is corroborative.

Conclusion

This paper advances the discourse on sensitivity and specificity, as well as affordances in terms of accessibility and affordability of eWBV with a view to re-evaluate utilization of WBV in clinical practice. This report highlights a clinical laboratory tool for evidence-based medical practice to manage cardiovascular phenomena, especially antiplatelet therapy. The results show that eWBV is generally more specific and less sensitive to common CVD phenomena. In particular, the laboratory tool showed a minimal sensitivity to thrombocytosis and higher than specificity to thrombocytopenia. The potential of adopting eWBV is for the benefit of patients' care with emphasis on affordances, reliability and validity. The long-term and universal utility is that patients who have haematocrit and serum protein level results i.e., from accessible routine laboratory tests, can have their WBV level determined.

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Data Availability

Data supporting the results reported in the manuscript have been previously published as referenced. However, any further request can be made through the authors

Conflicts of Interest

None to declare

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Research article

Assessment of the Analytical Performance of 14 Analytes using the Epoc® Blood Analysis System

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Objective: **To evaluate the analytical performance of Epoc® Blood Analysis System for 14 analytes (pH, pCO_2, pO_2, HCO_3^-, BE, sO_2, Na^+, K^+, iCa^{2+}, Cl^-, Glu, Lac, Crea and BUN)**

Material and Methods: **The coefficient of variation (CV%) was calculated based on a between-day replication study using internal quality control material at two concentrations. The relative mean difference (BIAS%) was calculated based on method comparisons of 53 to 55 arterial patient samples using ABL 835 Flex Blood Gas Analyzer (Radiometer) and Dimension Vista 1500 System (Siemens Healthineers). The total analytical error (TAE%) was estimated by calculation of the 95% confidence interval, which incorporates the observed CV% from the replication study and BIAS% from the method comparison study. Each analyte's precision, trueness and accuracy were assessed by comparing the observed CV%, BIAS% and TAE% to the analytical performance specifications (APS) from Westgard for imprecision (I%), bias (B%) and total allowable error (TE%), respectively. The analytical performance using the Epoc were considered acceptable in clinical settings if at least the minimum specifications for accuracy were achieved.**

Results: **pH, BE, K^+, Glu, Lac and BUN fulfilled the minimum specifications for precision, while pCO_2, HCO_3^-, Na^+, iCa^{2+}, Cl^- and Crea did not. pH, pCO_2, Na^+, K^+, Glu, Lac and BUN fulfilled the minimum specifications for trueness, while HCO_3^-, iCa^{2+}, Cl^- and Crea did not. pH, pCO_2, BE, K^+, Glu, Lac and BUN fulfilled minimum specifications for accuracy, while iCa^{2+} did not. No specifications were specified for pO_2 and sO_2.**

Conclusions: **pH, pCO_2, BE, K^+, Glu, Lac and BUN showed analytical performances considered acceptable for use in clinical settings, since at least the minimum specifications regarding accuracy were achieved. iCa^{2+} showed unacceptable analytical performance for use in clinical settings, whereas the results for HCO_3^-, Na^+, Cl^- and Crea were inconclusive.**

Key words: Point-of-care testing, POCT, Epoc, Method comparison, Biological variation

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Introduction

Epoc® Blood Analysis system (Siemens Healthineers, Erlangen, Germany) is a handheld point-of-care testing (POCT) device intended to be used by professionals in the healthcare setting as an *in vitro* diagnostic quantitative test for blood gasses, electrolytes, metabolites, haematocrits and other calculated parameters. The wide variety of tests available using the customized Epoc® BGEM Test Cards and rapid turnaround of results using only 92 µL of sample material makes it an attractive and useful device in emergency situations.[1]

Epoc's analytical performance against various reference methods has been assessed in prior studies.[2-14] Most of the studies were limited as they evaluated the clinical use of Epoc solely from the observed correlation between the Epoc and a reference method, whereas predefined analytical performance specification (APS) has only been included in four studies.[3-14] However, none of those studies included base excess (BE) or blood urea nitrogen (BUN), while saturated oxygen (sO_2) and standard bicarbonate (HCO_3^-) have only been included one and two times, respectively. Further, only four studies used ABL and Vista as the reference methods.[3,4,10,13]Therefore, there is still a need for further evaluation of the Epoc to get a better picture of the full capacity of the device. This study aimed to assess the analytical performance of the Epoc system to determine if the observed analytical performance can be considered acceptable for use in clinical settings according to the chosen assessment method.

Material and methods

Assessment criteria

The predefined APS used in this study was derived from intra- and interindividual biological variation taken from Westgard's database.[15] Epoc's precision, trueness and accuracy for each of the analytes was assessed by comparing the observed analytical performance to the predefined minimum, desired and optimal APS calculated based on the formulas by Fraser et al.[16] APS for pO_2 and sO_2 are not specified. The analytes precision was assessed by comparing the observed coefficient of variation (CV%) to the predefined APS expressed as imprecision (I%). The analytes trueness was assessed by comparing the observed relative mean difference (BIAS%) to the predefined APS expressed as inaccuracy (B%). The analytes accuracy was assessed by comparing the observed total analytical error (TAE%) to the allowable total error (TE%).

Sample collection

Fifty-five arterial whole blood samples were collected during two weeks in October 2020 from 16 different patients admitted at an intensive care unit (ICU) at a Danish Hospital. The samples were drawn from the patient's arterial lines by trained nurses using safePICO syringe with safeTIPCAP containing lithium heparin (Radiometer, Brønshøj, Denmark). No special permission was needed as the material was used in a quality assessment process of a new device prior to implementation. All patient sensitive information was anonymized before data was processed.

Assessment of precision

The observed CV% for each analyte was based on results from a replication study using Eurotrol GAS-ISE-Metabolite (Eurotrol Inc., Netherland) as quality control (QC) material at concentration levels one (L1) and three (L3). The QC material was analysed according to the operation instructions once a day for 10 consecutive days using the same Epoc device.[1] CV% was calculated as the standard deviation (SD) / mean x 100.

Assessment of trueness

The observed BIAS% was estimated based on a method comparison study between Epoc against ABL 835 Flex Blood Gas Analyser (ABL) and Dimension Vista® 1500 System (VISTA) as reference methods. First, the patient samples were analyzed using the ABL for all analytes except for Crea and BUN. Subsequently and within three minutes samples were analyzed using the Epoc system. Then leftover sample material was transferred from the syringe to VACUETTE® blood collection tubes with CAT

serum separator clot activator (Greiner Bio-One, Australia). Within 1.5 hours from ABL and EPOC analysis, the samples were centrifuged for 10 min / 2,500g at 20°C and hereafter stored at 2-8 °C. The samples were then analyzed for Crea and BUN the same or following day using VISTA. BIAS% was calculated as the relative mean difference (Epoc mean – reference method mean)/ average of methods (Epoc mean + reference method mean)/2) x 100.

Assessment of accuracy

The estimation of the observed TAE% was calculated as BIAS% (from the method comparison study) ± 1,96 x CV% (from the replication study) and represented the 95% confidence interval of the analytes analytical error.[15-18] For this calculation, the higher CV% value of L1 and L3 observed was used. The analytes overall analytical performance was considered acceptable in clinical settings if both TAE% limits fell within the minimum APS for TE%. The performance was considered unacceptable in clinical settings if both TAE% limits fell outside the minimum APS for TE% and inconclusive if the one TAE% limit fell within and one outside the minimum APS for TE%, since data was insufficient to assess the performance.[15-18]

Statistics

The statistical analysis and difference plots were performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). The Passing Bablok regression plots were performed using R Core Team (2021) (Vienna, Austria).

Results

Assessment of precision

The pH, BE, K^+, Lac and BUN met the optimal APS for I% (Table 1). pCO_2 met the optimal APS for I% for L1, whereas L3 exceeded minimum APS with as little as +0,02%. HCO_3^- met desired.

Table 1. The observed mean, standard deviation (SD) and variation coefficient (CV%) from the replication study using the Epoc system with two levels of QC material. Minimum, desired and optimal APS for I% were based on biological variation from Westgard and calculated according to Fraser et al.[15,16]

Analyte (unit)	Eurotrol QC L1		Eurotrol QC L3		APS for I%		
	Mean (SD)	CV%	Mean (SD)	CV%	Minimum	Desired	Optimal
pH	7.009 (0.012)	0.156	7.724 (0.012)	0.157	≤ 2.63	≤ 1.75	≤ 0.88
pCO2 (kPa)	9.36 (0.103)	1.10	2.94 (0.106)	**3.62**	≤ 3.6	≤ 2.4	≤ 1.2
pO2 (kPa)	9.00 (0.335)	3.745	25.27 (0.972)	3.848	N/A	N/A	N/A
HCO3- (mmol/L)	17.7 (0.32)	1.80	28.8 (1.33)	**4.61**	≤ 3.0	≤ 2.0	≤ 1.0
BE (mmol/L)	-13.45 (0.48)	3.56	9.3 (1.43)	15.43	≤ 57.3	≤ 38.2	≤ 19.1
sO2 (%)	80.6 (2.16)	2.69	99.9 (0.00)	0.00	N/A	N/A	N/A
Na+ (mmol/L)	115 (0.8)	**0.72**	162 (0.8)	0.49	≤ 0.53	≤ 0.35	≤ 0.18
K+ (mmol/L)	2.1 (0.00)	0.00	5.8 (0.07)	1.16	≤ 3.6	≤ 2.4	≤ 1.2
iCa2+ (mmol/L)	1.54 (0.023)	**1.52**	0.64 (0.016)	**2.52**	≤ 1.28	≤ 0.85	≤ 0.43
Cl- (mmol/L)	79.6 (0.94)	**1.18**	113.4 (2.01)	**1.77**	≤ 0.9	≤ 0.6	≤ 0.3
Glu (mmol/L)	1.9 (0.07)	3.73	14.6 (0.37)	2.50	≤ 4.2	≤ 2.8	≤ 1.4
Lac (mmol/L)	0.82 (0.045)	5.39	5.78 (0.391)	6.78	≤ 20.4	≤ 13.6	≤ 6.8
Crea (µmol/L)	69 (3.8)	**5.6**	340 (9.8)	2.9	≤ 4.5	≤ 3.0	≤ 1.5
BUN (mmol/L)	18.7 (0.37)	1.97	1.7 (0.05)	2.89	≤ 9.3	≤ 6.2	≤ 3,1

Values printed in bold indicate that the respective QC level exceeded the minimum APS defined for I%, thus showing unacceptable precision.

N/A: performance specification for the analyte not available. APS: Analytical performance specifications.

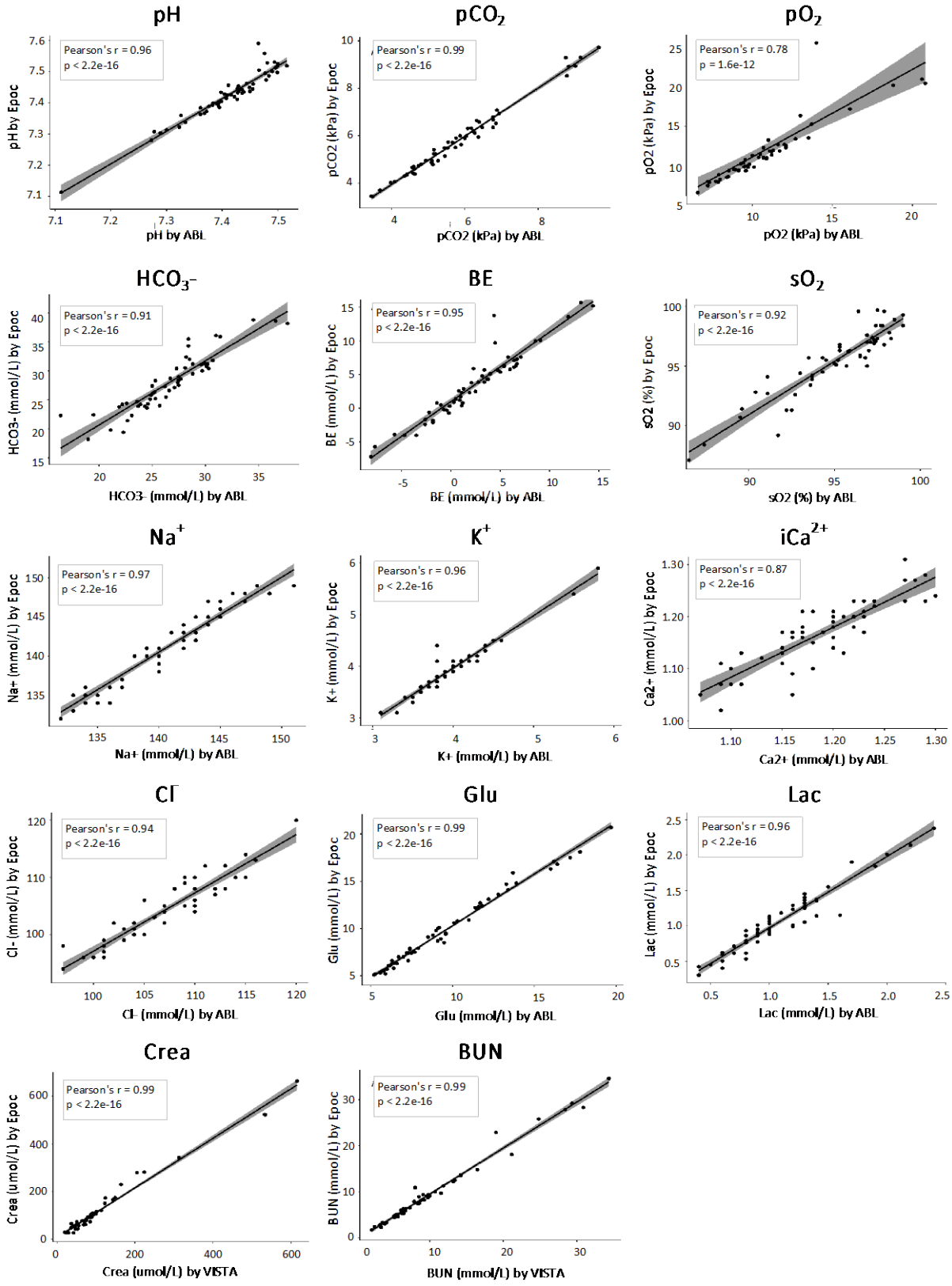

Figure 1. Passing Bablok regression with the reference measurements by ABL/VISTA on the x-axis as function of the Epoc measurements on the y-axis as well as Pearson's correlation coefficient (r) and p-value for each of the analytes.[22]

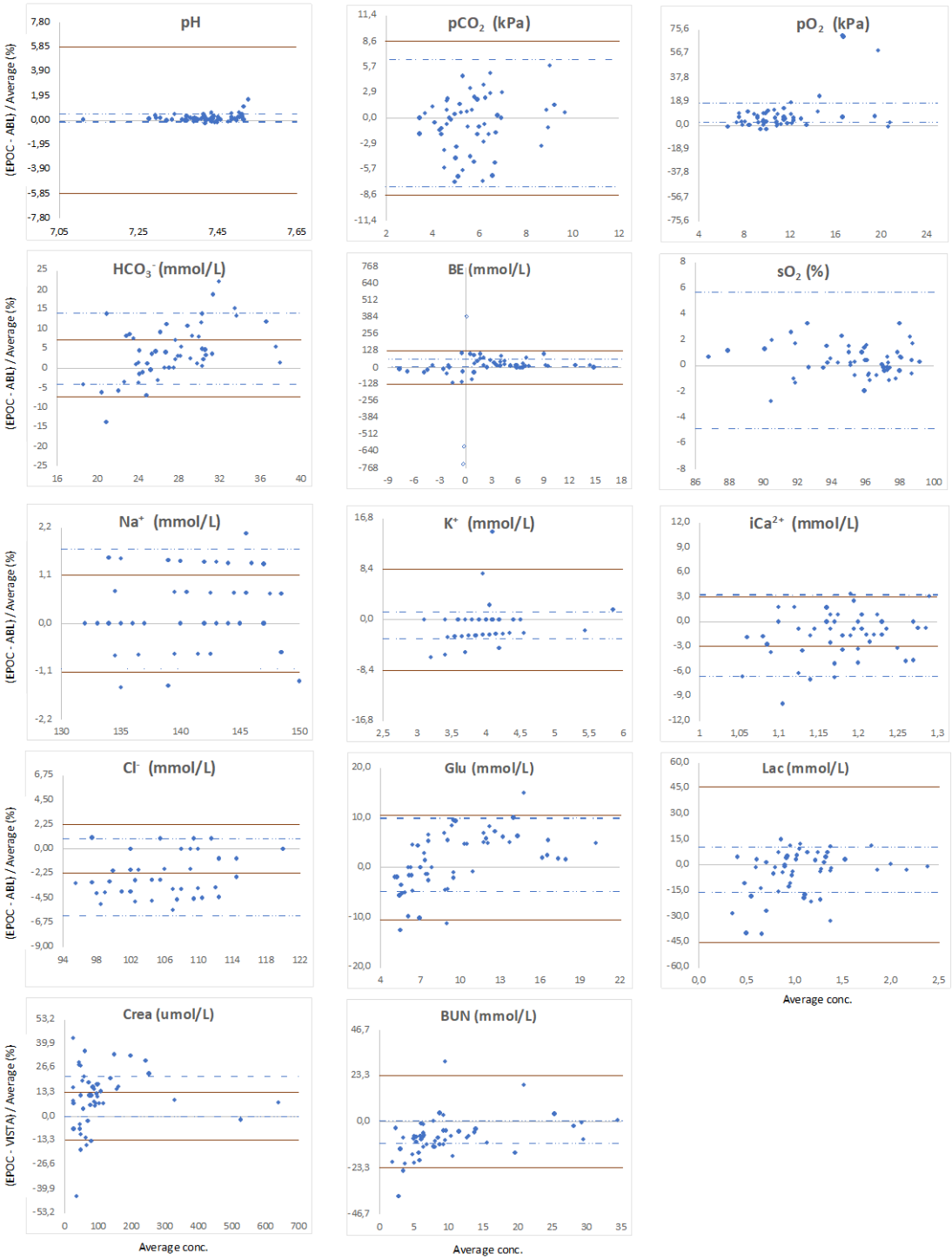

Figure 2. The relative difference between Epoc and the reference method as function of the average concentrations of both methods. Following analytes are included: pH, pCO2, pO2, HCO3-, BE, sO2, Na+, K+, iCa2+, Cl-, Glu, Lac, Crea and BUN.

The broken lines represent the observed TAE%, which was calculated using following formula: TAE% = BIAS% (from the method comparison) ± 1.96 x CV% (the higher CV% value of L1 and L3 from the replication study). The solid red lines represent the minimum APS for TE% from Westgard (15) and was calculated according to Fraser et al.16 If both TAE% limits (broken lines) fell within the minimum APS for TE%, then the analyte's analytical performance was considered acceptable in clinical settings.17 If both broken lines fall outside the minimum APS for TE%, then the analyte's analytical performance was considered unacceptable. If only one of the broken lines fall within the minimum APS for TE%, then the analyte's analytical performance was considered inconclusive.

APS for I% for L1, whereas L3 exceeded minimum APS. Glu met the minimum APS for I% for L1 and desired APS for L3. Na^+ met minimum APS for I% for L3, whereas L1 did not. Crea met desired APS for I% for L3, whereas L1 exceeded minimum APS. iCa^{2+} and Cl^- exceeded the minimum APS for I% for both L1 and L3

Assessment of trueness

Passing Bablok regression with the reference measurements by ABL/VISTA on the x-axis as function of the Epoc measurements on the y-axis as well as Pearson's correlation coefficient(r) for each of the analytes are shown in Figure 1.

The pH, pCO_2, K^+ and Lac met the optimal APS for B%. BUN met the desired APS for B%, while Na^+ and Glu met the minimum APS for B%. BE, HCO_3^-, iCa^{2+}, Cl^- and Crea do not meet any of the APS for B% (Table 2).

Assessment of accuracy

The estimated TAE% for pH met the optimal APS for TE%. TAE% for BE, K^+, Lac and BUN met the desired APS for TE%. pCO_2 and Glu met the minimum APS for TE%.TAE% for iCa^{2+} exceeded the minimum APS for TE%, whereas TAE% for HCO_3^-, Na^+, Cl^- and Crea overlapped with the minimum APS for TE%. (Figure 2 and Table 3).

Discussion

The assessment of Epoc's analytical performance using arterial blood showed that Epoc could be considered acceptable in clinical settings for pH, pCO_2, BE, K^+, Glu, Lac and BUN. These analytes fulfilled the defined minimum, desired or optimal APS for TE% based on Westgard's biological variations database.[15] The iCa^{2+} did not fullfill the defined APS for TE%, while HCO_3, Na^+, Cl^- and Crea showed inconclusive analytical performance. To our knowledge this is the first study to evaluate BE

Table 2. The observed mean difference (BIAS%) between Epoc and the reference method from the comparison study and the defined specifications for the trueness (B%). The minimum, desired and optimal APS for B% were based on biological variation from Westgard and calculated according to Fraser et al.15,16

Analyte (unit)	**Epoc** Mean (SD)	**Reference** Mean (SD)	**BIAS%** Epoc-Reference/(Average)	**APS for B%**		
				Minimum	Desired	Optimal
Epoc vs ABL 835 FLEX						
pH	7.425 (0.0791)	7.412 (0.0725)	0.184	±1.51	±1.01	±0.50?
pCO2 (kPa)	5.76 (1.491)	5.79 (1.467)	-0.53	±2.68	±1.79	±0.89
pO2 (kPa)	11.91 (4.200)	10.77 (2.935)	10.06	N/A	N/A	N/A
HCO3- (mmol/L)	27.91 (4.810)	26.55 (3.960)	**4.99**	±2.3	±1.6	±0.8
BE (mmol/L)	3.5 (5.12)	2.6 (4.70)	**35.6**	±32.85	±21.9	±10.95
sO2 (%)	95.6 (2.87)	95.2 (2.96)	0.39	N/A	N/A	N/A
Na+ (mmol/L)	141 (5.0)	141 (5.0)	0.33	±0.46	±0.31	±0.15
K+ (mmol/L)	4.0 (0.46)	4.0 (0.44)	-0.91	±2.77	±1.84	±0.92
iCa2+ (mmol/L)	1.17 (0.063)	1.19 (0.056)	**-1.68**	±0.96	±0.64	±0.32
Cl- (mmol/L)	104 (5.6)	107 (5.2)	**-2.61**	±0.72	±0.48	±0.24
Glu (mmol/L)	9.9 (3.98)	9.6 (3.61)	2.6	±3.51	±2.34	±1.17
Lac (mmol/L)	1.05 (0.447)	1.08 (0.426)	-2.7	±11.97	±7.98	±3.99
Epoc vs Dimension VISTA 1500						
Crea (µmol/L)	122 (129.6)	110 (123.8)	**10.7**	±5.95	±3.97	±1.98
BUN (mmol/L)	9.7 (7.53)	10.2 (7.47)	-5,1	±8.3	±5.5	±2.8

Values printed in bold indicate that BIAS% exceeded the minimum APS for B%, thus showing unacceptable trueness. N/A: performance specification for the analyte not available. APS: Analytical performance specifications. SD: Observed standard deviations from the method comparison.

Table 3. The overall analytical performance of each analyte using the Epoc System compared to the predefined minimum, desired and optimal analytical performance specifications (APS) for precision, trueness, and accuracy.

	Blood gases						Electrolytes				Metabolites			
	pH	pCO2	pO2	HCO3	BE	sO2	Na+	K+	iCa2+	Cl-	Glu	Lac	Crea	BUN
	-	kPa	kPa	mM	mM	mM	mM	mM	mM	mM	mM	mM	uM	mM
Precision(L1)	✓o	✓o	N/A	✓d	✓o	N/A	—	✓o	—	—	✓m	✓o	—	✓o
Precision(L3)	✓o	—	N/A	—	✓o	N/A	✓m	✓o	—	—	✓d	✓o	✓d	✓o
Trueness	✓o	✓o	N/A	—	—	N/A	✓m	✓o	—	—	✓m	✓o	—	✓d
Accuracy	✓o	✓m	N/A	?	✓d	N/A	?	✓d	—	?	✓m	✓d	?	✓d

Optimal APS met (✓o). Desired APS met (✓d). Minimum APS met (✓m). Defined APS not met (—). Inconclusive data (?). N/A: No APS for the analyte available.

and BUN using the Epoc system, whereas HCO_3^- and sO_2 have been evaluated two and one times before, respectively.[4,6,7]

The HCO_3^- is calculated as Log HCO_3^- = *pH* + *LOG* pCO_2 - *7.608*, thus making HCO_3^- dependent on pH and pCO_2's performance.[1] The analytical performance for HCO_3^- was concluded as inconclusive based on the APS from Westgard, although pH and pCO_2 showed acceptable analytical performance.[15] Previous studies concluded HCO_3^- analysis using Epoc to be equivalent to the reference method when using cappillary blood.[6,7] However, if this study adopted the target ±15% for TE% used by those studies, HCO_3^- would be considered acceptable in clinical settings.[6,7]

No APS were available for pO_2. The estimated TAE% for pO_2 was 2.52 to 17.61%. However, if the three outliers for pO_2 above the reference interval (>14.4 kPa) were removed from the dataset the estimated TAE% would be -2.69 to 12.40% as a result of the BIAS% improving with 5.21%. Thus, the TAE% would fall within the ±15% limits used by Kim et al. and Shin et al. and pO_2 could be considered acceptable in clinical settings.[6,7] The EPOC has a tendency of high measurements of O_2 in the range above the reference interval observed in prior studies.[3,7,14] Further examinations are needed in order to determine if this could be linked to a general characteristic of the Epoc in regard to pO_2 testing. The observed correlations (r=0.78) between Epoc and ABL for pO_2 was lower compared to prior studies (r=0.99) using the same methods.[3,4]

No APS based on biological variaiton was available for sO_2. The estimation of TAE% (-4.9 to 5,7%) for sO_2 was based on the highest CV% (QC Level 1). Using the lower CV% (QC Level 3) for the estimation of TAE% might have contributed to a precision that better reflected the values of the actual measurements, and thereby a more narrow TAE. Use of sO_2 in clinical settings can be considered acceptable if the laboratory is willing to accept a relative difference between EPOC and ABL of ±3.3% (equivalent to the maximum difference observed).

This study demonstrated a strong correlation (r=0.92) between Epoc and ABL for sO_2. This is close to the correlation (r=0.98) reported by Agarwal et al., which to our best knowledge is the only other study to include sO_2 using Epoc.[4] Although according to expert consensus, directly measured sO_2 such as with ABL (co-oximetry) are preferred for critically ill patients, whereas calculated sO_2 values should be interpreted with caution.[19,20]

The K^+ is the only electrolyte considered acceptable in clincial settings with all APS fullfilled at either optimal or desired APS. This is in line with previous studies.[2-5,9,10,14] None of the APS for iCa^{2+} were fullfilled, thus making the analyte unfit for use in clinical settings based on the defined APS. The observed correlation between Epoc and ABL (r=0.87) for iCa^{2+} was lower compared to the correlations

(r=0.98) reported in two prior studies.[3,4] Another study reported a weaker correlation between the methods (r=0.80) compared to this present study. However, that study used capillary blood and a limited sample size (n=8-10).[10]

Three of the previous studies concluded Na^+ to be useful based on TE% limits between ±2.0% and ±4%.[6,7,14] If those limits were adopted in this study, the conclusion would also be that Na^+ could be considered acceptable in clinical settings. The observed correlation in this present study for Na^+ (r=0.97) was stronger than the correlations found in other studies with ABL as the reference method (r=0.84-0.86).[3,4] The observed correlation for Crea (r=0.99) was in line with correlations reported in other studies across different reference methods.[4,10,13] However, only one of these studies concluded, that Crea was not fit for use in clinical settnigs and reported a larger BIAS% compared to the observed BIAS% in this present study.[8] In this study, a strong correlation between Epoc and the reference method for BE (r=0.95) and BUN (r=0.99) was observed. This is in line with prior studies using other available POCT devices with strong correlations.[21]

Limitations and Strengths

One limitation of this study, is that samples were analyzed on the ABL first and subsequently on the Epoc. This could potentially induce systematic bias to the measurements. Another limitation is that the used reference methods are not considered the golden standard for each individual analyte. The strength of this study was the number of samples being from admitted patients, thus making it possible to cover a wide range of concentrations with exception of the lower and higher concentration range for Glu and iCa^{2+}.

Conclusion

Of the 14 studied analytes pH, pCO_2, BE, K^+, Glu, Lac and BUN showed analytical performance acceptable to use in clinical settings according to the chosen assessment criteria. The iCa^{2+} showed unacceptable analytical performance, whereas HCO_3^-, Na^+, Cl^- and Crea showed inconclusive analytical performance compared to the APS for TE%. No APS for TE% are available for pO_2 and sO_2. However, the results showed that if the laboratory is willing to accept an estimated TAE% within ±17.61% for pO_2 and ≥ ±3.3% for sO_2, then the analytes can be considered acceptable in clinical settings.

Declarations of interest

All authors have no conflict of interest to declare.

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