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Special Report

Clinical Predictors of SARS-CoV-2 Testing Pressure on Clinical Laboratories: A Multinational Study Analyzing Google Trends and Over 100 Million Diagnostic Tests

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Laboratory Medicine 2021;52:311-314

DOI: 10.1093/labmed/lmab013

ABSTRACT

Objective: Evidence has shown that Google searches for clinical symptom keywords correlates with the number of new weekly patients with COVID-19. This multinational study assessed whether demand for SARS-CoV-2 tests could also be predicted by Google searches for key COVID-19 symptoms.

Methods: The weekly number of SARS-CoV-2 tests performed in Italy and the United States was retrieved from official sources. A concomitant electronic search was performed in Google Trends, using terms for key COVID-19 symptoms.

Results: The model that provided the highest coefficient of determination for the United States (R^2 = 82.8%) included a combination of searching for cough (with a time lag of 2 weeks), fever (with a time lag of 2 weeks), and headache (with a time lag of 3 weeks; the time lag refers to the amount of time between when a search was conducted and when a test was administered). In Italy, headache provided the model with the highest adjusted R^2 (86.8%), with time lags of both 1 and 2 weeks.

Conclusion: Weekly monitoring of Google Trends scores for nonspecific COVID-19 symptoms is a reliable approach for anticipating SARS-CoV-2 testing demands ~2 weeks in the future.

Keywords: laboratory medicine, SARS-CoV-2, diagnostic testing, infodemiology, laboratory management, COVID-19

The ongoing COVID-19 pandemic has placed unprecedented strain on clinical laboratories.[1] Despite significant efforts, the high demand for SARS-CoV-2 diagnostic tests, compounded by increased patient loads, shortages of reagents, and limited qualified personnel to perform the tests, has encumbered effective responses by laboratories to the pandemic.[1,2] A recent survey by the American Association of Clinical Chemistry concluded that the vast majority of worldwide laboratories are encountering serious challenges in obtaining reagents and test kits for routine SARS-CoV-2 diagnostics, along with shortages in qualified personnel to run the molecular assays.[3] Given that diagnostic testing with identification and isolation of patients who test positive is the most effective policy for preventing or containing local outbreaks[4] and that laboratory shortages are expected to continue well into 2021, the identification of predictors that could anticipate testing pressure on laboratories days or even weeks in advance would be helpful in preparing laboratories for a surge in demand and informing public health decision-making.

As previously shown, tracking the number of weekly Google searches for clinical symptom keywords, in particular the loss of taste and the loss of smell, correlated strongly with the number of weekly cases of patients diagnosed with COVID-19 2 weeks later.[5] However, the demand for laboratory tests may be impacted by many factors other than the number of actual patients with SARS-CoV-2, such as the circulation of common cold viruses, influenza, or

Abbreviation:

USA, United States of America.

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even seasonal allergies, all of which can mimic COVID-19 symptoms. As such, in this retrospective, multinational study, we aimed to assess whether the demand for SARS-CoV-2 tests could be accurately predicted by Google searches for key COVID-19 symptoms.

Materials and Methods

The weekly number of SARS-CoV-2 tests performed in Italy and the United States (USA) was retrieved by searching the official website of the Italian National Institute of Health (IstituSuperiore di Sanità) and the Centers for Disease Control and Prevention. Data was retrieved from both websites for a period between March 1 (ie, the beginning of the outbreak in the 2 countries) and December 20, 2020. A concomitant electronic search was carried out in Google Trends (Google Inc., Mountain View, CA), using the Italian and English terms for the most common symptoms described by COVID-19 patients: *febbre* (fever), *tosse* (cough), *dispnea* (dyspnea), *perdita olfatto* (olfactory loss), *perdita gusto* (taste loss), and *mal di testa* (headache). A weekly Google Trends score was obtained for each keyword, reflecting the cumulative number of Google searches during the previous 7 days. Data from Google Trends were retrieved for each country and keyword independently.

Cross-correlation analysis was conducted to identify time lags that provided the highest possible correlations between the Google Trends searches and SARS-CoV-2 testing (ie, the amount of time between when a search was conducted and when a test was administered). Time-series linear regression was performed for each search term to evaluate its predictive value in estimating the weekly number of SARS-CoV-2 tests. To adjust for the varying accessibility and number of available diagnostic tests over the course of local outbreaks, a numeric variable representing the epidemiologic month was included in each model. Further variable selection was based on the previous cross-correlation analysis. Model performance was assessed using adjusted R^2 and graphical analysis. All statistical analysis was performed using R software (The R Project for Statistical Computing, Vienna, Austria).

The study was conducted in accordance with the Declaration of Helsinki, under the terms of relevant local legislation. This analysis was based on electronic searches in unrestricted, publicly available repositories, so that no informed consent or ethical committee approvals were needed.

Results

A total of 114,936,353 SARS-CoV-2 diagnostic tests, 25,366,124 tests from Italy and 89,570,229 from the USA, were analyzed over the study period. The results of the cross-correlation analysis are presented in **Table 1**. In the data from Italy, fever, cough, and dyspnea provided the highest correlations with no time lag, headache and smell loss hit their peak cross-correlation coefficient when there was a time lag of 2 weeks, and taste loss had its highest correlation when there was a time lag of 3 weeks. In the USA, fever, cough, taste loss, and dyspnea provided the highest correlation with the number of SARS-CoV-2 tests when the Google Trends search had a time lag of 1 week. The term *headache* reached its peak correlation when there was a 4-week time lag, and smell loss had its highest correlation when there was no time lag.

Time-series linear regression analysis (**Table 2**) showed that the effects of the Google Trends search series for fever (a lag of 2 weeks), headache (a lag of 3 weeks), cough (a lag of 2 weeks), and dyspnea (lags of both 1 and 2 weeks) were each significant when adjusted for the monthly trend of an increase in tests in the USA, and all provided adjusted R^2 values of >77%. Neither smell nor taste loss showed significant effects in the number of weekly tests. The model that provided the highest coefficient of determination for this data, R^2 = 82.8%, included a combination of cough (a lag of 2 weeks), fever (a lag of 2 weeks), and headache (a lag of 3 weeks) in addition to the monthly trend.

Similar results were found using the Italian data: Both fever and cough, when there was a 2-week time lag, had significant effects on the number of weekly SARS-CoV-2 tests and provided adjusted R^2 values of 76.4% and 76.9%, respectively, when adjusted for the monthly trend. The same was observed for smell loss, when there was a 2-week time lag, producing an adjusted R^2 of 80.7%. Taste loss also had significant effects on the number of weekly tests, at both 1- and 3-week time lags, with an adjusted R^2 of 84.3%. The term *headache* provided the model with the highest adjusted R^2 (86.8%), when there was a time lag of both 1 and 2 weeks. The Google Trends search series for

Table 1. Cross-Correlation Analysis Between Weekly Number of SARS-CoV-2 tests in the USA and Italy with Google Trends Scores for Suggestive Symptoms

Search Term	Optimal Time Lag	Cross-Correlation Coefficient	*P* Value
USA			
Fever	–1	–0.643	<.001
Cough	–1	–0.632	<.001
Headache	–4	–0.538	<.001
Smell loss	0	0.345	.027
Taste loss	–1	0.386	.013
Dyspnea	–1	–0.494	.002
Italy			
Fever	0	–0.118	.4499
Cough	0	–0.091	.5601
Headache	–2	0.426	.006
Smell loss	–2	0.306	.050
Taste loss	–3	0.408	.009
Dyspnea	0	–0.396	.011

Table 2. Time-Series Linear Regression Analysis for Weekly Number of SARS-CoV-2 Tests in the USA and Italy with Google Trends Scores for Suggestive Symptoms

USA				Italy			
Variable	**Coefficient**	**SE**	***P* Value**	**Variable**	**Coefficient**	**SE**	***P* Value**
Month	371514.413	37173.475	<.001	Month	124197.56	10271.921	<.001
Taste loss (lag: 2 weeks)	–4604.735	4542.435	.317	Taste loss (lag: 1 week)	3264.247	1174.573	.009
R^2 = 0.744				Taste loss (lag: 3 weeks)	4430.998	1172.683	.001
				R^2 = 0.843			
Month	368141.424	34967.842	<.001	Month	130744.074	11033.681	<.001
Smell loss (lag: 2 weeks)	–6205.882	4995.789	.222	Smell loss (lag: 2 weeks)	6025.338	1289.034	<.001
R^2 = 0.736				R^2 = 0.807			
Month	286427.622	38786.036	<.001	Month	139022.829	12484.744	<.001
Fever (lag: 2 weeks)	–18475.442	6310.324	.006	Fever (lag: 2 weeks)	6359.982	1887.627	.002
R^2 = 0.787				R^2 = 0.764			
Month	284906.054	34068.422	<.001	Month	140929.918	12454.365	<.001
Cough (lag: 2 weeks)	–18397.907	4754.003	<.001	Cough (lag: 2 weeks)	5794.213	1645.429	.001
R^2 = 0.814				R^2 = 0.769			
Month	292549.787	35925.514	<.001	Month	125283.07	9150.488	<.001
Headache (lag: 3 weeks)	–29846.516	10117.8591	.006	Headache (lag: 3 weeks)	7519.16	2735.181	.009
R^2 = 0.773				Headache (lag: 2 weeks)	8828.424	2753.355	.003
				R^2 = 0.868			
Month	305947.217	31327.072	<.001	Month	142714.572	16100.452	<.001
Dyspnea (lag: 1 week)	–15789.835	7301.935	.038	Dyspnea (lag: 2 weeks)	3697.781	2514.167	.150
Dyspnea (lag: 2 weeks)	–17374.205	7050.279	.01878	R^2 = 0.707			
R^2 = 0.814							
Month	305214.726	38859.025	<.001	…	…	…	…
Cough (lag: 2 weeks)	–52445.943	18270.325	.007	…	…	…	…
Fever (lag: 2 weeks)	50882.5341	23382.953	.037	…	…	…	…
Headache (lag: 3 weeks)	–25093.088	10181.607	.019	…	…	…	…
R^2 = 0.828				…			

SE, standard error.

Lab Medicine 2021;52;311–314
DOI: 10.1093/labmed/lmab013

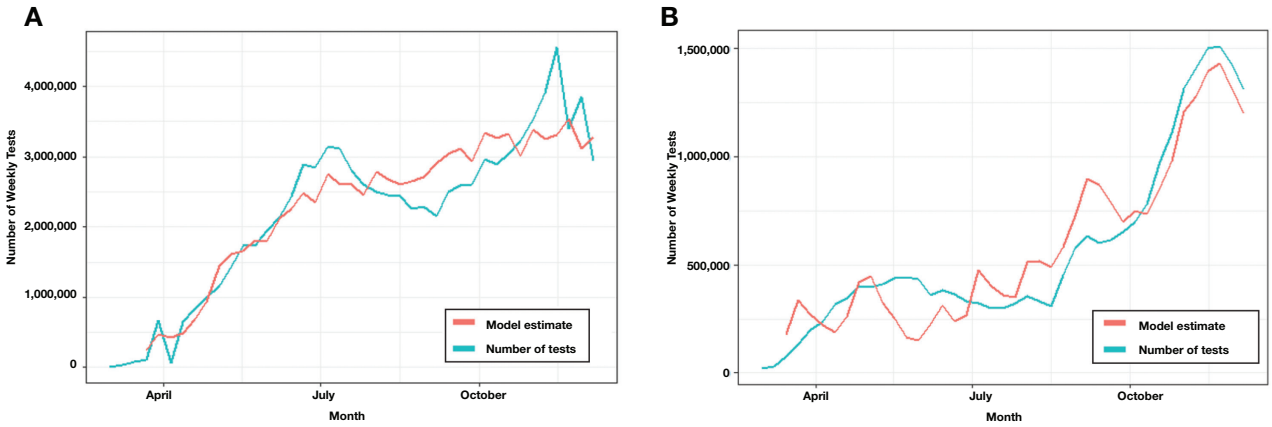

Figure 1

Highest adjusted R^2 time-series linear regression results for the number of weekly SARS-CoV-2 tests in the USA **(A)** and Italy **(B).**

the term *dyspnea* had no significant effect on the weekly test numbers. The 2 models with the highest adjusted R^2 for each country are represented in **Figure 1**.

Discussion

The results of our analysis show that Google Trends data can anticipate the demand for SARS-CoV-2 diagnostic tests. Overall, the results were relatively consistent between both countries. Interestingly, we observed that nonspecific symptoms (fever, headache, and cough) were the best predictors for the number of weekly diagnostic tests 2 to 3 weeks after the search was conducted. This result contrasts with data previously observed for predicting the number of patients diagnosed weekly with COVID-19 using the same symptom keywords, in which specific symptoms, ie, taste loss and smell loss, were the best predictors.[5] We suspect that this contrast is because nonspecific COVID-19 symptoms can result from numerous other conditions, which may also have seasonal variability, such as the common cold or allergies, all of which are likely to lead individuals to seek SARS-CoV-2 testing.

Our study was limited by its retrospective design and the fact that access to COVID-19 testing, availability of test kits, number of tests performed, and public health testing programs may have all changed over the course of the pandemic. We controlled for this by using the month as a variable in the linear regression.

Conclusion

Based on the results of our analysis, we recommend that clinical laboratories and public health officials monitor weekly Google Trends scores for nonspecific symptom searches, which can obtained freely and easily in seconds and on a more localized, regional level. A 2- to 3-week lag for the keywords *fever, headache,* and *cough* to anticipate testing demands would enable clinical laboratories to stock up on reagents and plan staffing accordingly and to inform public health officials to set up pop-up testing sites and allocate additional resources to upcoming hot spots, as appropriate. **LM**

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Overview

A High-Level Overview of the Regulations Surrounding a Clinical Laboratory and Upcoming Regulatory Challenges for Laboratory Developed Tests

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Laboratory Medicine 2021;52:315-328

DOI: 10.1093/labmed/lmaa086

ABSTRACT

Objective: Regulations for clinical laboratories in the United States are complex. The goal of this review is to improve the clarity of laboratory-developed test (LDT) regulation to facilitate innovation.

Methods: A literature and regulation review of current legislation for compliance by U.S. clinical laboratories was performed, and examples of the steps to implement LDTs within compliance with the regulatory environment are shared.

Results: Many federal and state jurisdictions are critical to the functionality of a laboratory in addition to upcoming potential promulgation of the Verifying Accurate Leading-Edge IVCT Development Act. Increased regulation, although imperative to maintain consistent, high-standard clinical care, could mean additional costs for developers and healthcare while also hindering innovation.

Conclusion: An extensive discussion of proposed regulations for LDTs needs to occur. Laboratory testing requires the sustained use of innovative methods at a cost that will permit continued, timely, uninterrupted high-quality service.

Keywords: regulations, CLIA, laboratory developed test, innovation, VALID Act, clinical laboratory

A common quote at medical conferences and industry workshops alike is that approximately 70% of all medical decisions are based on laboratory test results.[1] Laboratories provide accurate and precise evidence, which is crucial for disease diagnosis, follow-up, therapy management, and estimation of risk factors. Because of their capacity for providing powerful insights, clinical laboratories have significant responsibilities in the healthcare system. Therefore, it should come as no surprise that clinical laboratories are highly regulated in the United States. Clinical laboratories are present in healthcare institutions of all sizes, from small hospital clinical laboratories to stand-alone specialty private practices or reference laboratories to large academic medical centers. Different types of tests are offered in each of these settings with variable complexity, running the spectrum from point-of-care testing to intricate genetic sequencing; all of the tests are subject to a variety of different regulatory requirements.

Abbreviations:

LDT, laboratory developed test; SSA, Social Security Act; CLIA'67, Clinical Laboratories Improvement Act of 1967; PHSA, Public Health Service Act; QC, quality control; PT, proficiency testing; CDC, Centers for Disease Control & Prevention; CLIA'88, Clinical Laboratory Improvement Amendments of 1988; CMS, Centers for Medicare & Medicaid Services; COW, Certificate of Waiver; NYS, New York State; WS, Washington State; DOH, department of health; LQA, Laboratory of Quality Assurance; IVD, in vitro diagnostic; FDA, U.S. Food and Drug Administration; PMA, premarket approval; EUA, emergency use authorization; IVCTs, in vitro clinical tests; CSF, cerebral spinal fluid; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; HPLC, high-performance liquid chromatography; OIR, Office of In-Vitro Diagnostics and Radiological Health; CLSI, Clinical and Laboratory Standards Institute.

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Overview of U.S. Clinical Laboratory Testing Regulation

The first set of regulations, published in 1966 under Titles 18 and 19 of the Social Security Act (SSA),[2] required that

laboratories serving as providers in the Medicare program be subject to quality standards established by the Secretary of Health, Education, and Welfare.[3] The Clinical Laboratories Improvement Act of 1967 (CLIA'67)[4] was passed, and Section 353 of the Public Health Service Act (PHSA)[5] required laboratories that sent specimens via interstate commerce to be subject to regulation by the federal government. The CLIA'67 primarily covered independent and hospital laboratories and included requirements for quality control (QC) and proficiency testing (PT). The program was implemented by the Centers for Disease Control (CDC; currently called the Centers for Disease Control & Prevention). During this time, the federal government regulated laboratories under 2 programs: Medicare/Medicaid for independent laboratories through the SSA and CLIA'67 for laboratories conducting interstate testing through the CDC. In 1974, these 2 programs adopted each other's standards, with both programs having personnel, QC, and PT requirements. A *Wall Street Journal* article published in 1987[6] highlighted the shortcomings of the Pap smear testing, whose personnel were overworked, undersupervised, and poorly paid. Although the instances of abuses were isolated and far from the practices of most laboratories, pathologists and cytotechnologists feared that the public would view the negative portrayal as representative of the entire clinical testing profession. This incited the need for CLIA to require additional quality assurance measures for laboratories, including proof of laboratory certification.[6]

On October 31, 1988, Congress enacted the Clinical Laboratory Improvement Amendments of 1988 (CLIA'88), Public Law 100–578.[7] The CLIA '88 replaced Section 353(e)[5] of the PHSA, as enacted by CLIA'67.[4] Although the CLIA had 2 updates throughout the years, it continues to be referred to as CLIA'88 in legislation as a federal program for the certification and oversight of clinical laboratory testing. The assessment and evaluation of clinical regulatory agencies, and their influence on the laboratory testing environment, will provide insight into the complex nature of CLIA and the continued capacity for certified CLIA laboratories to provide a wide variety of approved tests for the healthcare industry. References and links with complete information on regulations are available in the reference list. A historical timeline of major regulatory events related to clinical laboratory testing is represented in **Figure 1**.

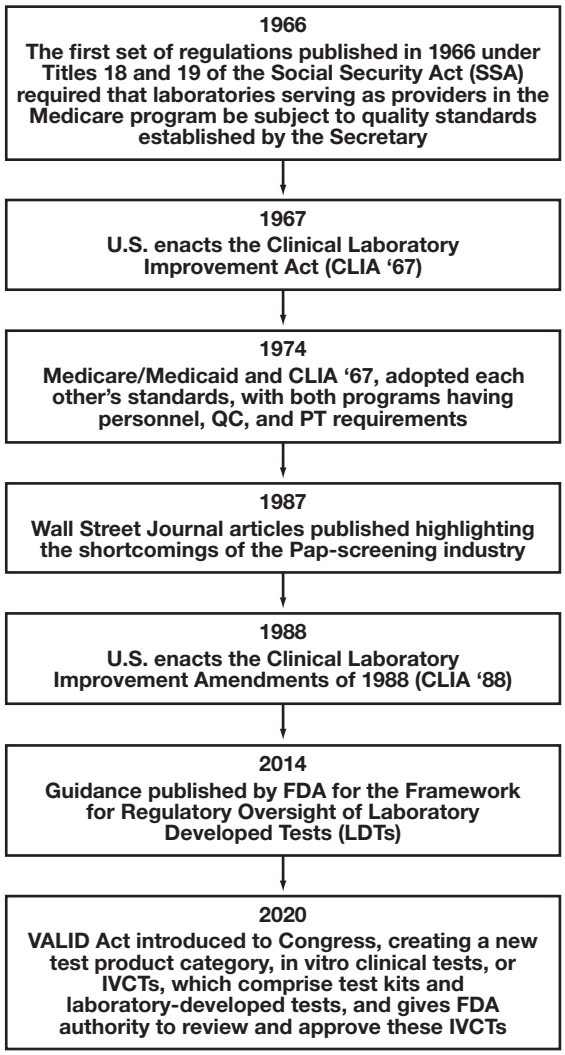

Figure 1

Historical timeline of major regulatory events related to clinical laboratory testing.

Roles of Governing Regulatory Agencies

Centers for Medicare & Medicaid Services and CLIA

The Centers for Medicare & Medicaid Services (CMS) regulates all human clinical laboratory testing performed in the United States through CLIA. This process requires clinical

laboratories to be certified by their state and by CMS before they can accept human specimens for diagnostic testing.[8] Laboratories can obtain multiple types of CLIA certificates, based on the kinds of diagnostic tests they conduct: (i) a Certificate of Waiver (COW) allows a laboratory to perform only waived tests; (ii) a Certificate for Provider-Performed Microscopy Procedures is issued to a laboratory where a physician, midlevel practitioner, or dentist performs microscopy procedures and allows the laboratory to perform waived tests; (iii) a Certificate of Registration is a temporary permission to perform waived and nonwaived (moderate- and/or high-complexity) laboratory testing until the laboratory is determined to be in compliance with the CLIA regulations through an inspection; (iv) a Certificate of Compliance is issued to a laboratory after an inspection that finds the laboratory to be in compliance with all applicable CLIA requirements; and (v) a Certificate of Accreditation is issued to a laboratory on the basis of the laboratory meeting the accreditation standards of a deemed status organization approved by CMS.[9] The different types of CLIA certificate examples are shown in **Table 1**.

The CMS also collects CLIA user fees, conducts inspections, enforces regulatory compliance, monitors laboratory performance on PT, and approves PT programs.[10] Approved accreditation organizations under CLIA include the College of American Pathologists, the Joint Commission on Accreditation of Healthcare Organizations, the American Association of Blood Banks, the American Association for Laboratory Accreditation, the Accreditation Association for Hospitals and Health Systems/Healthcare Facilities Accreditation Program, and the American Society for Histocompatibility and Immunogenetics (**Figure 2**).[11]

As of 2019, New York State (NYS) and Washington State (WS) were both deemed to have CLIA-exempt status from the CMS. In the context of CLIA, this status simply means that laboratories in either state will receive a state permit for testing rather than a CLIA certificate. Although all states must fully comply with CLIA, they may choose to enforce a state law or regulation that is equally or more stringent than the CLIA-mandated rules.[12] Both NYS and WS have regulatory authority over clinical laboratory testing written into state law that is overseen by their state department of health (DOH). All clinical laboratories located in or accepting specimens from NYS must hold a state clinical laboratory permit in appropriate testing categories and be subject to NYS law governing clinical laboratory testing.[13] Similar to NYS, under the WS Medical Test Site licensure program, the Office of Laboratory Quality Assurance (LQA) of the DOH issues licenses or COWs, conducts onsite surveys of nonwaived medical test sites, verifies corrections of deficiencies, and monitors PT results. The majority of reference laboratories serve NYS clients, and focus throughout this article yields to the NYS DOH Clinical Laboratory Evaluation Program. Further information regarding the WS LQA authority and responsibilities can be found on the WS DOH Web site.[14]

Table 1. CLIA-Certified Laboratory Categorization and Requirements

FDA Categorization	Requirements	Description	Inspection Required?
Waived	CLIA COW Follow manufacturer instructions	Simple tests Pose no reasonable risk of harm with an incorrect result Approved for home or over-the-counter use	No
Provider-performed microscopy procedures; moderate complexity	CLIA certificate of provider-performed microscopy procedures	A physician, midlevel practitioner, or dentist performs microscopy procedures while also allowing the laboratory to perform waived tests.	No[52]
Nonwaived; moderate complexity	CLIA Certificate of Registration followed by a Certificate of Compliance or Certificate of Accreditation Must meet CLIA quality system standards (PT, QC/QA), personnel requirements	Complicated tests or test system	Yes
Nonwaived; high complexity	CLIA Certificate of Compliance or Certificate of Accreditation Must meet CLIA quality system standards (PT, QC/QA), personnel requirements	Complicated tests or test system	Yes

CLIA, Clinical Laboratories Improvement Act; COW, Certificate of Waiver; PT, proficiency testing; QA, quality assurance; QC, quality control.
Laboratories have different certificates and requirements according to the complexity of testing performed. Full information available at https://www.cdc.gov/clia/test-complexities.html.

DOI: 10.1093/labmed/lmaa086

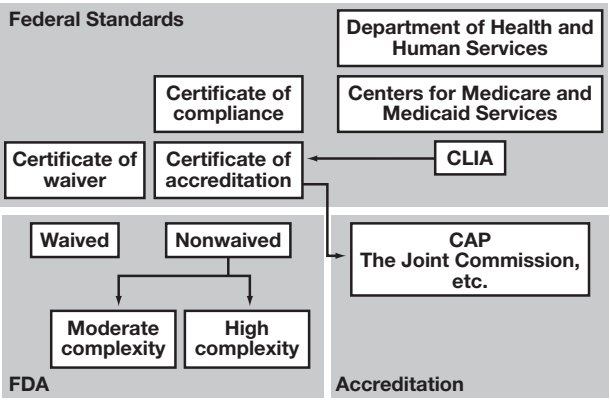

Figure 2

Roles of governing regulatory agencies for the clinical laboratories in the United States.

Classification of Laboratory Tests

The majority of laboratory tests in use today are manufactured, marketed, and sold as kits to numerous laboratories as in vitro diagnostic (IVD) kits, which are reviewed, approved, and regulated by the U.S. Food and Drug Administration (FDA). The FDA utilizes several pathways to review and approve IVDs, including the premarket approval (PMA) pathway for the highest-risk assays and devices, the 510(k) pathway for clearance of an assay or device that is substantially equivalent to one already marketed, and other more specialized pathways, such as for those deemed to be FDA-exempt or for those granted emergency use authorization (EUA). The EUA enables the FDA to facilitate the availability of specific, new, unapproved tests that can be used during an epidemic such as with the Ebola and Zika viruses or during a pandemic such as with SARS-CoV-2[15] to diagnose, treat, or prevent serious or life-threatening conditions when there are no adequate, approved, or available alternatives. The FDA categorizes IVD tests by complexity, based upon the level of risk that the test will provide incorrect information or cause patient harm if performed incorrectly, or if inaccurate results are reported.[10]

In addition to a laboratory being able to perform a test using an FDA-cleared kit per manufacturer instructions and EUA, the CLIA allows a laboratory to develop and perform a test within a single laboratory or organization; these are referred to as laboratory developed tests (LDTs). An FDA-approved assay may become an LDT if the laboratory makes a significant modification to the manufacturer's instructions not cleared or approved by the FDA. According to 42 CFR §493.1253, the federal standard for the establishment and verification of performance specifications, any laboratory that modifies an FDA-cleared or approved test system, or introduces a test system not subject to FDA clearance or approval (including methods developed in-house and standardized methods such as text book procedures), or uses a test system in which performance specifications are not provided by the manufacturer must, before reporting patient test results, establish for each test system the performance specifications for the following performance characteristics, as applicable.[16] One particular modification of an FDA-approved method would involve the use of a different matrix, body fluid, or specimen type. This variation is the most commonly associated modification with clinical laboratories across the healthcare industry. For example, if serum was the only specimen type approved for testing in the FDA-approved test system, then the analysis of urine would result in an assay being classified as an LDT. This procedure has several ramifications for laboratories, including that LDTs require additional validation requirements and are automatically high-complexity tests related to compliance with the CLIA, which includes more stringent requirements for testing, personnel education, and training, such as the requirement for testing personnel to have a specific number of credit hours in chemistry and biology (CLIA §493.1489 standard; testing personnel qualifications).[17]

Complexity of Laboratory Tests

The FDA determines a test's CLIA complexity by reviewing the package insert instructions and uses a "scorecard" to classify a test as being of moderate or high complexity.[10] The criteria assessed include (i) the amount of scientific and technical knowledge required to perform the test; (ii) the amount of training and experience necessary; (iii) the stability and reliability of materials and reagents; (iv) the complexity of operational steps; (v) the calibration, QC, PT, or other quality assurance activities required; (vi) the amount of system troubleshooting and equipment maintenance required; and (vii) the amount of interpretation and judgment necessary to perform preanalytic, analytic, and postanalytic processes,[10] as shown in **Table 2** (42 CFR §493.17).[18]

The manufacturer of a test categorized as being of moderate complexity may request categorization of the test as waived through a CLIA Waiver by Application submission to the FDA. When a test is categorized as waived, it may be performed by laboratories with a COW after sufficient evidence is provided to the FDA under regulation 42 CFR

Table 2. CLIA Criteria to Categorize Assay Complexity

		CLIA Categorization Criteria						
Score	**Sample Reasoning for Each Score for Each Category**	**Knowledge**	**Training and Experience**	**Reagents and Materials Preparation**	**Characteristics of Operational Steps**	**Calibration, QC, and PT Materials**	**Test System Troubleshooting and Equipment Maintenance**	**Interpretation and Judgment**
1	A	Minimal	Minimal	Reagents and materials are generally stable and reliable	Operational steps are either automatically executed (such as pipetting, temperature monitoring, or timing of steps), or are easily controlled	Calibration materials are stable and readily available	Test system troubleshooting is automatic or self-correcting, or clearly described or requires minimal judgment	Minimal interpretation and judgment are required
	B	On-the-job instruction	Limited experience required	Reagents and materials are prepackaged, or premeasured, or require no special handling, precautions or storage conditions		Quality control materials are stable and readily available—external proficiency testing materials, when available, are stable	Equipment maintenance is provided by the manufacturer, is seldom needed, or can easily be performed	Resolution of problems requires limited independent interpretation and judgment
3	A	Specialized scientific and technical knowledge	Specialized training	Reagents and materials may be labile and may require special handling to assure reliability	Operational steps in the testing process require close monitoring or control, and may require special specimen preparation, precise temperature control or timing of procedural steps, accurate pipetting, or extensive calculations	Calibration materials, if available, may be labile	Troubleshooting is not automatic and requires decision-making and direct intervention to resolve most problems	Extensive independent interpretation and judgment are required
	B		Substantial experience may be necessary	Reagents and materials preparation may include manual steps such as gravimetric or volumetric measurements		Quality control materials may be labile, or not available—external proficiency testing materials, if available, may be labile	Maintenance requires special knowledge, skills, and abilities	Resolution of problems requires extensive interpretation and judgment

CLIA, Clinical Laboratories Improvement Act; PT, proficiency testing; QC, quality control.
Scores for the 7 criteria are added together. Tests with a score of 12 or less are categorized as being of moderate complexity and those with a score above 12 are categorized as being of high complexity.[10] A score of 2 is assigned when the characteristics for a particular test are intermediate between the descriptions listed for scores of 1 and 3.[10]

§493.15(c). The list of waived tests is constantly growing, and these tests are usually performed at the point of care.[10] The complexity of testing performed at a specific location determines the type or scope of permit or certificate that a laboratory must hold. Laboratories must obtain the appropriate certificate for the complexity of testing offered for the CLIA site and ensure that personnel and other applicable requirements in the federal regulation are met.

LDT Regulation

Although the FDA claims regulatory oversight of IVDs, it has typically exercised enforcement discretion over LDTs such that LDTs do not routinely require clinical laboratories to submit for PMA. Instead, the FDA typically defers to the CMS for laboratory oversight through the CLIA, although that paradigm may change in the future. The FDA believes that CLIA is primarily focused on laboratory operations and may not adequately assure the safety and effectiveness of LDT design, development, and verification/validation.[19] The CLIA requires that laboratories assess analytic performance for a new LDT—ie, proof that the device accurately and reproducibly detects the analyte(s) of interest, but this process is generally only independently reviewed during routine biennial inspections and after the test is already available for patient testing[19] by the CLIA or other accrediting agencies. Routine inspections typically focus on analytic validation (accuracy of the detecting biomarker) and not clinical validation (accuracy of identifying, measuring, or predicting a clinical condition). In some cases, LDT review may not happen during routine inspections; be loose or an inspector may not have the necessary knowledge to assess the LDT. Accordingly, there may not be assurance that an LDT is clinically relevant, an area of significant concern for the FDA. In addition, although accrediting agencies may require adverse event reporting, the CLIA itself does not, making it difficult for regulators to detect LDTs that are inaccurate, ineffective, or unsafe.[19]

The NYS law differs from the CLIA in that clinical validity must be shown for an LDT. Explicit test-specific approval is not required for tests designated as FDA-cleared, approved, or exempt; however, LDTs must be approved by NYS before offering the assay for patient testing.[20] The state utilizes a risk-based evaluation of LDTs, facilitated by a risk attestation form that directs the assignment of the LDT into a low-, moderate-, or high-risk category.[21] Risk categories are based on 3 criteria: whether the methodology is well understood within the laboratory, whether the test is a key determinant, and the patient impact of an inaccurate result. A well-established methodology suggests that the test is a standard of care or that the laboratory has received approval for other tests using the same methodology. A key determinant is a test result that provides essential information to diagnose and/or indicate a greater likelihood of developing a disease or condition and to indicate eligibility for a specific treatment. Once a risk category is assigned, low-risk tests are typically approved, moderate-risk tests are typically conditionally approved pending full review, and high-risk tests are not approved for NYS patient testing until a full review is completed.

The categorization of testing under NYS DOH regulation, comparable to CMS/CLIA methodology, is determined by the intended clinical use of the testing, the test method employed, and the related category-specific Certificate of Qualification required for the laboratory director and/or assistant director responsible for the testing. The laboratory director/assistant director is responsible for the design of LDTs, the initial validation of all tests, the monitoring and verification of ongoing performance, the training and competency program, and the provision of consultation/interpretation to clients of the laboratory regarding the test. If the individual designated as the laboratory director does not qualify for a Certificate of Qualification in each permit category, then the director may designate 1 or more individuals holding a certificate in the required category to serve as director for the category. The technical supervisor or technical consultant, who works under the directives of the laboratory director, is usually the holder of the Certificate of Qualification for the NYS-certified laboratory and has expertise in the methodologies employed in the laboratory for clinical testing (**Table 3**).[22]

Operational Considerations for Clinical Laboratory Tests

Validation Requirements for Test Implementation

Each laboratory that introduces an unmodified, FDA-cleared test system must show that it can obtain performance specifications comparable to those established by the manufacturer for the following performance characteristics for each specimen type: precision, accuracy, reportable

Table 3. Laboratory Director Responsibilities in a High-Complexity CLIA-Certified Laboratory

CLIA Role	NYS Role	Responsibilities
Laboratory director Training required: 1. Licensed MD, DO, and board certification in clinical and/or anatomic pathology of both by the ABP or AOBP 2. PhD in chemical, physician, or clinical laboratory science program with board certification from the ABB, ABCC, ABFT, ABHI, ABMLI, ABMGG, ABMM, or NRCC	Laboratory director Training required: 1. Licensed MD, DO and board certification in clinical and/or anatomic pathology of both by the ABP or AOBP 2. PhD in chemical, physical, or clinical laboratory science program with board certification from the ABCC, ABFT, ABMLI, or ABMM	Responsible for overall laboratory operations and management of laboratory: —employment of personnel who are competent to perform test procedures —record and report test results promptly, accurately, and proficiently —compliance assurance with all applicable regulations —delegation of authority so that all duties are performed For NYS, the laboratory director is assigned based on the laboratory address, so multiple laboratory directors are allowed if the laboratory has more than 1 location.
Technical supervisor or technical consultant	Assistant director, Certificate of Qualification holder	Responsible for technical and scientific oversight of laboratory. Not required to be onsite at all times when testing is performed but must be available to laboratory on as-needed basis to provide supervision. Responsibilities include: —appropriate test selection, with adequate method validation —development of SOPs and maintenance records, including QA, QC, and personnel documentation —supervising enrollment in CMS-approved proficiency testing programs —ensuring that PT specimens are tested and reviewed by appropriate staff —carrying out corrective actions recommended by CLIA laboratory director, PT programs, or other regulatory/accrediting agencies —ensuring and supervising development of effective QA and QC programs —documenting remedial actions and identifying systems for maintenance by keeping appropriate documentation on test systems —overseeing laboratory personnel performance and competency —planning for training or continuing education needs

ABP, American Board of Pathology; ABB, American Board of Bioanalysis; ABCC, American Board of Clinical Chemistry; ABFT, American Board of Forensic Toxicology; ABHI, American Board of Histocompatibility and Immunogenetics; ABMGG, American Board of Medical Genetics and Genomics; ABMLI, American Boards of Medical Laboratory Immunology; ABMM, American Board of Medical Microbiology; AOBP, American Osteopathic Board of Pathology; CLIA, Clinical Laboratories Improvement Act; CMS, Centers for Medicare & Medicaid Services; NRCC, National Registry of Certified Chemists; NYS, New York State; PT, proficiency testing; QA, quality assurance; QC, quality control.
Training required for clinical chemistry laboratory is shown. For specialty laboratory testing, additional training, certification and years of experience may be required.

range of test results for the test system, and verify that the manufacturer's reference intervals (normal values) are appropriate for the laboratory's patient population.[16]

Laboratories may develop an LDT or may need to modify an FDA-cleared/approved test kit to meet their needs. If a laboratory deviates from the manufacturer's package insert/instructions for use, eg, by increasing specimen stability or adding an unapproved specimen type, then it is no longer using the test per manufacturer's instructions; it is operating outside the bounds of FDA approval obtained by the manufacturer, potentially affecting test performance. As a result, such modifications may move the test from being considered FDA-cleared to an LDT. In addition, beyond validating precision, accuracy, reportable range, and reference interval, a laboratory will need to assess analytical sensitivity (limit of detection/limit of quantification) and analytical specificity (selectivity and interference testing), and several sets of experiments are required before the LDT can be implemented in a clinical laboratory (typical examples are shown in **Table 4**). If more than 1 specimen type is utilized for testing, then a laboratory is required to separately address performance specifications for each specimen type. A fully validated test may be implemented in a clinical laboratory after studies have been conducted and have met predefined specification criteria.[23]

Quality System of a Clinical Laboratory

The quality system of a laboratory must be constructed to assess and continuously improve the performance and delivery of its services to meet patient needs[24] through

DOI: 10.1093/labmed/lmaa086

Table 4. Clinical Laboratory Test Validation Parameters

Parameter	Definition	Typical Set of Experiments	References
Precision	The closeness of agreement between independent test results obtained under stipulated conditions Repeatability Within lab precision	Assessing 5 replicates per day for 5 days per level of quality control material or pooled sera to assess whether the precision of an assay meets acceptability criteria	EP05—Evaluation of Precision Performance of Quantitative Measurement Procedures EP15—User Verification of Precision and Estimation of Bias
Accuracy	The closeness of the agreement between the result of a measurement and a true value of the measurement.	Most often accomplished by performing a method comparison between the new assay and the reference method using an appropriate number of samples across the dynamic range of the assay (ranging from 20 – 120 samples)	EP10—Preliminary Evaluation of Quantitative Clinical Laboratory Measurement Procedures EP12—User Protocol for Evaluation of Qualitative Test Performance
Reference intervals		The clinical laboratory must verify or establish reference intervals. Verifying reference intervals from an FDA-approved kit may be done using several different approaches. CLSI suggests the measurement of a minimum of 20 healthy individuals so long as no more than two fall outside the proposed limits.[45] If validation fails or if reference intervals have not been established, another common approach is to run 120 samples from healthy individuals in order to establish lab- and population-specific reference intervals.	EP28—Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory
Reportable range	The clinical lab assesses the linearity or analytical measuring range (AMR) of an assay taking into account eventual dilutions of the sample which can extend the AMR.	The laboratory runs a series of levels across the AMR stated in the performance claims of the method. This must include both the maximum concentration tested and minimum concentration within the specified limits of the assay. Serial- or pooled-dilutions can be performed using patient samples or commercially-available linearity material can also be used.	EP06—Evaluation of the Linearity of Quantitative Measurement Procedures
Analytical sensitivity[a]	Analytical sensitivity represents the smallest amount of substance in a sample that can accurately be measured by an assay; ability to detect very low concentrations of a given substance in a biological specimen, often referred to as the limit of detection (LOD) (EP15). LOD is the actual concentration of an analyte in a specimen that can be consistently detected ≥ 95% of the time (EP15).		EP17—Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures
Analytical specificity[a]	Analytical specificity refers to the ability of an assay to measure on particular organism or substance, rather than others, in a sample. An evaluation of the potential for interference that may occur when a specimen is introduced to endogenous substances like hemoglobin, bilirubin, lipemia, rheumatoid factor, common medications, or blood tube serum separators, is important for method verification studies because these substances may inhibit the analyte from binding to the intended target.		EP07—Interference Testing in Clinical Chemistry

[a]*Experiments only needed for LDTs. Complete information available in each of the CLSI documents described here and in CLSI EP19, 2nd edition.*[46]

ongoing monitoring that identifies, evaluates, and resolves problems for all phases of the testing process (42 CFR §493.1200).[25] For each test system, the laboratory is responsible for having control procedures that monitor the accuracy and precision of the complete analytic process, as stated in 42 CFR §493.1256.[26] In addition, PT programs managed by states or professional organizations provide a means to show the quality of laboratory performance and

are utilized to cover reportable ranges per testing category and any instrument operational qualification and performance qualification requirement(s).[12] Software requirements and capabilities should be implemented to facilitate reference intervals and specifications and, whenever possible, allow broader data analyses.

Laboratories conducting moderate- and high-complexity testing must participate in a CLIA PT program for certain tests, and a list of regulated analytes is available where PT is mandatory and imprecision requirements are established for test performance. In addition, PT offers a laboratory performing nonwaived tests a way to measure performance and verify its accuracy and reliability. A CMS-approved PT program sends laboratories a set of specimens approximately 3 times a year or on a scheduled basis. Laboratories must test the PT specimens just like patient specimens and report the results to the PT program. Laboratories are not allowed to share specimens or results across different CLIA numbers for a PT specimen. The PT program grades the results using the CLIA grading criteria and returns them to laboratories so that they can assess how accurately and reliably they performed testing. Such programs receive annual and ongoing regulatory reviews by the CMS and accreditation organizations.[27] Many LDTs will not have an externally available PT program, so a laboratory will need to develop an alternative assessment of performance to show ongoing quality, such as retesting blind specimens, specimen exchange with other laboratories performing testing for the same analyte, or chart reviews of patients' clinical conditions.

Personnel Requirements

Personnel are the most essential part of any workplace, including the laboratory. Ensuring that there are enough properly trained people is one of the most important functions of the laboratory director. The CLIA requires that before testing patient specimens, all personnel must receive the appropriate training (or retraining, if necessary) and be competent to perform testing reliably and accurately; training must include awareness of device defects that may occur because of improper job performance or that an employee may encounter during routine job functions.[28] Technical staff must be trained before performing testing. New technical staff must be assessed for competency at 6 months before entering into the normal annual competency assessment schedule. Qualification requirements for laboratory personnel performing nonwaived testing are defined in 42 CFR §493, Subpart M, for each test complexity category.

Laboratory Test Catalog

Test information must be available to physicians who order clinical laboratory tests and to patients who are tested. A searchable electronic laboratory test catalog is an ideal mechanism to convey critical information about specimen requirements, test methodology, interpretation, and contact information for further questions. A laboratory test catalog may also organize tests according to their classification, ie, FDA-cleared tests or LDTs.

The laboratory is responsible for ensuring that tests remain appropriately classified after implementation. The laboratory should utilize its standard operating procedures for the tests, package inserts for all active ingredient reagents, and instrument manuals, as applicable, to aid in classification. Test classification is performed to determine the type of validation required, evaluate personnel requirements, identify appropriate comments for the laboratory test catalog, ensure that the correct comments appear on patient reports, and aid in the submission of appropriate materials to the NYS DOH for test approval, as applicable.

Current Landscape and Challenges for LDTs

In early February 2020, the U.S. Department of Health and Human Services recognized the novel coronavirus, SARS-CoV-2 (causing the disease COVID-19), as a public health emergency. As a result, any laboratory wishing to offer diagnostic testing would need EUA approval from the FDA before implementing testing for the virus. The EUA process effectively hindered implementation of validated COVID-19 testing, delaying patient diagnosis. In response to the increasing severity of the pandemic and the urgent need for expanded testing capability across the country, the FDA modified its EUA process. The agency allowed high-complexity CLIA laboratories to develop, validate, and implement polymerase chain reaction–based COVID-19 tests and allowed for 15 business days to submit the required

Lab Medicine 2021;52;315–328 323
DOI: 10.1093/labmed/lmaa086

documentation—a review process parallel with the implementation of the test.[29] The expedited EUA process serves as a potential precedent that could be leveraged in the Verifying Accurate Leading-Edge IVCT Development (VALID) Act (discussed in the next paragraph). A significant concern in the laboratory community is the negative impact that FDA oversight will have on the time it takes to bring an assay to market. Parallel review for tests that are not high risk, functionally a conditional approval pathway, may mitigate some of those concerns.

The VALID Act's Potential Implications for the Regulatory Oversight of the Clinical Testing Industry

Discussion draft legislation released at the end of 2018, the VALID Act, seeks to place IVDs and LDTs under the same regulatory umbrella, regulated by the FDA, collectively referring to these devices as in vitro clinical tests (IVCTs) and regulated by the FDA.[30,31] The bill was introduced by U.S. House and Senate lawmakers on March 5, 2020, but it has yet to be acted on by Congress. The potential for FDA oversight of LDTs described in the VALID Act in its current draft format, as of July 2020, will require clinical laboratories to proactively assess and address all regulatory principles in relation to their testing catalog. If the VALID Act had been implemented when the SARS-CoV-2 pandemic began, then it could have significantly delayed the time to market of LDTs for the virus.

For brand-new LDTs, certain features of the VALID Act should be of particular interest to IVCT developers, which would include clinical laboratories: (i) the proposal of a technical certification program, which could allow eligible developers to market certain IVCTs without a full submission to the FDA[30,31]; (ii) a grandfathering provision for tests on the market before the enactment of the VALID Act[30,31]; (iii) a multiyear transition period to accommodate high-volume testing laboratories with extensive laboratory testing menus[30,31]; and (iv) the framework for a third-party review program, because the FDA does not currently have the necessary resources to review the many thousands of LDTs on the market.[30,31]

LDTs: Examples of Clinical Impact for Personalized Care

We share below 3 examples of tests validated for use as a LDT before implementation in a clinical laboratory. The first example highlights a modification to the pre-analytic processing of the specimen, the second is a modification to the specimen type for the test, and the third was developed and validated entirely by the laboratory.

An LDT with a Preanalytic Modification: C1q Complement, Functional Serum

The complement system is composed of a cascade of > 50 soluble and cell-bound proteins. Measurement of complement activity in human sera can provide important information in the diagnosis of complement immunodeficiencies or of autoimmune diseases associated with dysregulation of the complement pathways. The most common laboratory test for complement activity is the classical pathway function, also known as the CH50, which measures the function of the classical pathway of the complement from its first recognition molecule until its final step, the assembly of the membrane attack complex. There are FDA-approved tests for CH50 measurement, such as a liposome immunoassay for the quantitative determination of total complement activity (ie, the CH50) in human serum.[32] This FDA-approved test was modified by mixing patient serum with a C1q-deficient serum before testing. This modification changed the intended use of the assay: After mixing with a C1q-deficient serum, the assay can measure the specific C1q deficiency, if present. A C1q deficiency is associated with increased incidence of immune-complex disease (eg, systemic lupus erythematosus, the most common manifestation of C1q deficiency[33]; polymyositis; glomerulonephritis). Inherited deficiency of C1q is rare.

An LDT with a Specimen-Type Modification: Kappa Immunoglobulin Free Light Chains, Spinal Fluid

Measurement of kappa and lambda free light chains in serum specimens is FDA-approved for their diagnostic, monitoring, and prognostic roles in the evaluation of monoclonal gammopathies. The test is part of the diagnostic criteria for multiple myeloma requiring therapy since 2014,[34] is part of the therapy response criteria defined by

the International Myeloma Working Group,[35] and aids in stratifying patients at higher risk of progression to malignancies when they are initially diagnosed with a premalignant condition such as monoclonal gammopathy of undetermined significance.[36] In the past decade, the use of free light chains measurement in cerebral spinal fluid (CSF) has been considered as a replacement test for oligoclonal bands to aid in the evaluation of multiple sclerosis. The measurement of kappa immunoglobulin free light chains in CSF has similar sensitivity and specificity to oligoclonal bands, with significant technical advantages to the laboratory such as a short turnaround time for results and automation.[37] The use of the kappa free light chain reagents in CSF changes the intended use of the test and requires a classification as an LDT, with a significant need for additional validation studies by the performing laboratory.

An LDT with a New Technique: The Example of MASS-FIX

In the study of plasma cell malignancies, M-protein immunotyping by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) of serum specimens offers patients an unprecedented way to monitor a monoclonal protein (M-protein) that is traditionally found using serum protein electrophoresis or immunofixation.[38,39] The finding of an M-protein is suggestive of monoclonal gammopathy, a group of conditions ranging from premalignant to malignant. Once the condition is diagnosed, patients are commonly monitored for the rest of their lives. The LDT assay uses MALDI-TOF MS to qualitatively identify the immunoglobulin heavy chain type (gamma, alpha, or mu) and light chain type (kappa or lambda) in the serum specimen, replacing isotyping of the M-protein by immunofixation, and was given the name MASS-FIX to differentiate it from the electrophoretic-based immunofixation. The utility of the test has been well established over time, but a novel technique has been employed for the detection of M-proteins, replacing the electrophoretic system. Although the primary interpretation finding is the same as the traditional immunofixation electrophoretic method, there are new features that can be observed on MASS-FIX such as the observation of glycosylated light chains. It has been shown that patients with glycosylated light chains have 14 times more chances of having amyloidosis than patients without glycosylated light chains,[40] and hence the new test can aid in risk stratification for the early detection of amyloidosis. Furthermore, because the molecular mass of the M-proteins are identified, it is possible to monitor the specific M-protein over time and distinguish between a therapeutic monoclonal antibody such as daratumumab, isatuximab, or elotuzumab from an endogenous disease–causing clone.[41] Although this particular test has been developed and fully validated by a clinical laboratory as an LDT and its performance characteristics were determined in a manner consistent with CLIA requirements, the test has not been cleared or approved by the FDA; however, these new capabilities have significant advantages when compared to the traditional FDA-approved immunofixation method for identifying M-proteins.

Regulatory Status of Clinical MS Instrumentation

As an example of an LDT, the MASS-FIX test system employs a mass spectrometer instrument with MALDI-TOF. Currently, high-performance liquid chromatography (HPLC) and MS designed for clinical use are Class I exempt devices under the respective regulations in 21 CFR §862.2260 and 21 CFR §862.2860[42-44] (**Table 5**). This classification means that the manufacturer does not have to obtain FDA approval or clearance before marketing, although the instruments must be manufactured under quality systems regulations (Quality System Regulation, 21 CFR Part 820[44,45]) and be labeled for IVD use (Section 809.10[b]).[44,46] Note, however, that the Class I status

Table 5. IVD Devices, Classification According to the FDA

	Class I	Class II	Class III
Complexity and risk	Simple, low risk	More complex, moderate risk	High risk, very complex
Example	Blood glucose meter	Immunoassay	Next-generation sequencing
FDA pathway	Exempt	510(k)	PMA
Process controls	General controls	General and special controls	General and special controls, clinical data (eg, clinical trial)
Marketing	FDA registered or listed	FDA cleared	FDA approved

FDA, U.S. Food and Drug Administration; IVD, in vitro diagnostic; PMA, premarket approval.
This is a summation of the most common submission pathways; every device and pathway will have unique considerations. Alternative pathways include De Novo, Humanitarian Device Exemptions, and Custom Device Exceptions, to name a few.

DOI: 10.1093/labmed/lmaa086

applies only to the instrument itself—it does not apply to an assay that is performed using the instrument unless the assay itself is also Class I exempt. When an instrument is used to measure a specific analyte, the instrument plus the associated reagents are classified as a test system and the system as a whole is reviewed on the basis of the risk of the assay. For example, a Class III HPLC-MS cancer screening IVD will require that the HPLC-MS instrumentation also be reviewed as a Class III device, and its use in the assay is subject to PMA. Moreover, for both Class II and Class III devices, subsequent modifications to the assay, instrumentation, or software require additional submissions for FDA review.[44]

A device utilizing MALDI-TOF MS was approved by the Office of In-Vitro Diagnostics and Radiological Health (OIR) at the Center for Devices and Radiological Health at the FDA in 2012 for the identification of microorganisms cultured from human specimens. The technological differences between HPLC-MS/MS and MALDI-TOF MS are significant; furthermore, the innovative efforts to expand the intended use of MS identification capabilities present new regulatory challenges. It is critical to appreciate that the FDA's definition of an assay or device may not include just the HPLC and the MS elements but the entire device workflow, from specimen collection and preparation to generation and reporting of the result, including software, associated testing (panel testing), and databases, depending on the manufacturer's provided intended use.[44] An assay for vitamin D using liquid chromatography-MS/MS was FDA-approved by an IVD company in 2017,[47] opening a path for other assays to follow.

Whereas OIR plans to continually address the increasing novelty and importance of MS-based protein and peptide IVDs in the clinical laboratory setting, current Clinical and Laboratory Standards Institute (CLSI) documentation—specifically C50-A, Mass Spectrometry in the Clinical Laboratory: General Principles and Guidance[44,48] and C62-A, Liquid Chromatography-Mass Spectrometry Methods[44,49]—do not address devices that would measure proteins and peptides, although there is a specific draft recommendation in the works by CLSI committees to address this gap, tentatively named "Quantitative Measurement of Proteins and Peptides by Mass Spectrometry (C64)".

Many of these topics have been discussed at length in the literature, but the development of common standards and methods has been difficult for the research and clinical communities. Ongoing discussion of these topics in the context of IVDs could assist device developers and manufacturers in continuing to bring these types of IVDs to the clinic.

The lack of predicates is a challenge that OIR faces regularly with novel devices and should not be viewed as an impediment to clearance or approval of MS-based IVDs.[44] The lack of recognized international reference materials and control materials and the potential lack of clinical diagnostic gold standards are complicated issues for all types of technologies, including MS, which will benefit from further discussion.[44]

LDT Validations Oversight

The MASS-FIX, an LDT under CLIA, requires a full submission of all validation experiments to the NYS DOH because of its novelty. Before a full review is conducted by the NYS DOH, NYS physicians must submit test requests with a restricted laboratory permit (approval) from the NYS DOH by completing a nonpermitted laboratory test request approval form.[50] One-time approvals (ie, a restricted laboratory permit) are then issued to the requesting physician or laboratory and to the laboratory requested to perform the test.[50] With each issuance of a restricted laboratory permit, laboratories are reminded of the permit and test-specific approval requirements.[50] After NYS DOH approval of the LDT is granted, NYS physicians can refer testing to the performing laboratory. The C1q complement function measurement in serum and kappa immunoglobulin free light chains in spinal fluid, although still LDTs under CLIA, are modifications of FDA-approved assays and require a more limited submission to NYS DOH, following the modifications to approved assay submission.[20] If the VALID Act was currently approved, and the LDTs were brand new to the laboratory, a submission of the LDT validation summary to the FDA or an agency deemed accredited to perform review of LDTs on behalf of the FDA would be required before test implementation.

Future Challenges

The future of clinical laboratory regulation is uncertain, but what is certain is that laboratories need to maintain

consistency in the way they perform assays, develop and validate methods, and comply with current quality management standards. The maintenance and sustainability of robust protocols and quality management systems will prove to be invaluable if the clinical laboratory industry is faced with increased LDT regulations. In addition, if third-party organizations such as the NYS DOH are further utilized for the risk-based review of new clinical test submissions, then the onerous task of reviewing them could be alleviated by accrediting additional organizations.[51] Furthermore, the implementation of a precertification process could allow an entire test group to be precertified by the FDA or a surrogate organization.[51] Although regulations can be designed to increase flexibility and efficiency in the operation of regulations and approval processes, their primary purpose is to ensure the safety of the patient population while being faced with rapid medical innovation and unmet clinical needs. The fostering of innovation needs to be maintained, and new legislative proposals need to fully reflect the needs, complexity, and resources involved in LDT development and operation in a clinical laboratory environment.[30,31] **LM**

Acknowledgments

The authors thank David L. Murray, MD, PhD, and Curt Hanson, MD, pathologists and laboratory directors in the Department of Laboratory Medicine and Pathology at the Mayo Clinic, for their critical review of this manuscript and helpful insights. The Mayo Clinic has filed intellectual property (IP) for the MASS-FIX assay described in this report as an LDT. The IP is licensed to an IVD company with intentions to bring the assay forward as an FDA-approved test system. None of the authors are named inventors in the IP. All authors declare no conflict of interest for this report.

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Science

Significant Operational Improvements with Implementation of Next Generation Laboratory Automation

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Laboratory Medicine 2021;52:329-337

DOI: 10.1093/labmed/lmaa108

ABSTRACT

Objectives: To investigate the benefits and challenges of introducing next generation chemistry and coagulation automation.

Methods: We replaced the Roche modular preanalytic system attached to Roche Cobas 6000 analyzers with the Roche 8100 preanalytical line attached to the Roche Cobas 8000 and Stago STA R Max analyzers. The system included 2 add-on buffers (AOBs) for automated specimen archival and retrieval and primary-tube specimen processing. We measured turnaround time (TAT) from specimen receipt to result for chemistry and coagulation tests before, during, and after system implementation. TAT for add-on tests was also measured.

Results: We completed the system implementation during a 17-month period using existing laboratory space. The TAT for chemistry, coagulation, and add-on tests decreased significantly ($P < .005$, $P < .001$, and $P < .005$, respectively). We encountered several challenges, including barcode-label errors, mechanical problems, and workflow issues due to lack of bidirectional track for coagulation testing.

Conclusions: Next generation laboratory automation yielded significantly shortened and less-variable TAT, particularly for add-on testing. Our approach could help other laboratories in the process of implementing and configuring automated systems.

Keywords: automation, preanalytical line, turnaround time, add-on testing, clinical chemistry, chemistry systems

Automated systems have become indispensable for hospital-based and commercial chemistry and hematology laboratories.[1–12] Several studies[1–12] have been published on the benefits of automation, such as smaller specimen-volume requirements,[1,3,4,6,8] reduction in errors, improved productivity, increased throughput, and more robust performance.[6] Yu et al[9] demonstrated reductions in process-handling steps, testing footprint, and the number of technical staff members needed to operate the system. There was also less variability in turnaround time (TAT), allowing the percentage of specimens/tests that met TAT goals to remain unchanged, despite increasing test volume. Laboratory automation can also create more-efficient workflows and improve safety through implementation of positive specimen identifications and tracking.[2,7,10,12] Preanalytical processes are particularly important to consider when implementing automation. It is therefore recommended to optimize the flow and quality of specimens entering the laboratory before automating core laboratory processes.[13–15] More recently, laboratories have also achieved efficiencies after introducing automated systems for routine microbiology tests.[16–19]

Implementing laboratory automation can be challenging because it requires tight project coordination, space

Abbreviations:

TAT, turnaround time; BWH, Brigham and Women's Hospital; LIS, library information system; EHR, electronic health record; MPA, modular preanalytical; AOB, add-on buffers; PT, prothrombin time; INR, international normalized ratio; IQR, interquartile range; ED, emergency department; IT, internet technology; CI, confidence interval; CK, creatine kinase; TSH, thyroid-stimulating hormone; LDH, lactate dehydrogenase; NT-proBNP, N-terminal pro-brain natriuretic peptide; DI, diabetes insipidus; NA, nonapplicable

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renovation, relocation of instrumentation, infrastructure upgrades, informatics support, and comprehensive test validation. Limited studies, such as one by Leung,[20] have described the challenges of introducing next generation laboratory automation using the existing laboratory space, a frequent problem for many hospital-based laboratories. This study aimed to describe the process and challenges of implementing next-generation automation using existing laboratory space, including its impact on TAT for high-volume chemistry, coagulation, and add-on tests.

Methods

Study Site and Automation

Brigham and Women's Hospital (BWH) is a 793-bed tertiary-care hospital located in Boston, MA. The BWH Core Laboratory processes approximately 3 million specimens per year. Our laboratory information system (LIS) is Sunquest (Sunquest Information Systems, Inc.), our middleware is Data Innovations (Data Innovations Inc.), and our electronic health record (EHR) is Epic (Epic Healthcare Systems).

Our laboratory decided to renovate the processing area and upgrade the existing automated laboratory system for several reasons, including the age of the system, frequency of downtime, increased volume of testing, new features available with the system upgrade, and opportunity to attach coagulation analyzers to the new automated line. We formed a multidisciplinary team composed of project managers (BWH and those from the vendor), operational, technical and medical directors, supervisors, information systems (BWH and vendor) and BWH facilities to design, stage, validate, monitor, and implement this system upgrade.

System Upgrade

Before the upgrade, our laboratory was using the Roche Cobas modular preanalytical (MPA) system and the Cobas 6000 chemistry system consisting of 3 CCE lines (c601, c601, e602; all manufactured by F. Hoffman-La Roche Ltd). The MPA centrifuges were set to a 5-minute spin at 1885g. Specimens for coagulation testing were not processed on the MPA but instead were sorted, manually centrifuged for 10 minutes at 1500g, and transported to 1 of the 3 Stago instruments by laboratory technicians.

In January 2017, the laboratory began its transition to the Roche Cobas 8100 preanalytical system, the Cobas 8000 chemistry analyzer series, and STA R Max coagulation analyzers (Diagnostica Stago, Inc.). The Cobas 8100 consists of an input buffer with stat port, 3 centrifuges (7-minute spin at 2780g to accommodate coagulation testing), 1 destopper, 1 barcode labeler, 1 aliquoter, 1 output buffer, 1 specimen-check module, 2 restoppers (1 for screw tops and 1 for phlanges), and 2 add-on buffers (AOBs). Compared with the previous MPA line, the Cobas 8100 accommodates 1 additional centrifuge, has AOBs, offers a specimen volume detector, connects bidirectionally to chemistry analyzers (but not coagulation analyzers), and has double the throughput (800 vs 400 specimens/hour).

We introduced 3 analytical Cobas 8000 chemistry lines (2 lines containing one c702, one c502, and one e602, as well as 1 line containing one c702 and 2 e602) and 3 STA R Max coagulation instruments. The Cobas 8000 Analyzer series was implemented on September 5, 2017. The Cobas 8100, including the AOB, was connected to the chemistry analyzers and implemented on December 12, 2017. The coagulation analyzers were connected unidirectionally to the 8100 line on May 30, 2018. Due to space and budgetary constraints, the automated specimen storage and retrieval unit was not purchased.

Add-on Testing

Briefly, add-on requests are placed by providers in the EHR and are available in the LIS.[21] The laboratory is alerted of the request by receiving a printout in the specimen processing area. A laboratory technician then searches the LIS for an available specimen (factoring in appropriate specimen type and stability for the requested tests) and adds the order to that specimen in the system. Specimens are sent to the AOBs (nonrefrigerated temporary storage on the automated line) via the bidirectional Roche track after the tests have been performed on the analyzers. Based on our capacity of 2000 tubes and weekday daily volume of 5000 tubes, we estimated that specimens will remain in the AOB for approximately 8 hours until they are transferred to longer-term refrigerated storage. If the pertinent specimens are being stored in the AOBs at the time the technician places the add-on order

in the LIS, the 8100 analyzer will automatically retrieve the specimen and direct it to the appropriate analyzer; if it is not in the AOBs, technicians manually retrieve the specimen from long-term refrigerated storage. We compared the TAT for specimens retrieved vs not retrieved from the AOB. We also calculated the percentage of add-on tests ordered within 8 hours of the original specimen receipt and percentage of requests we were unable to fulfill. Lastly, we identified the most frequent tests ordered as add-ons across all time periods.

TAT Analysis and Statistical Analysis

We assessed the TAT for tests performed on the Cobas 8000s (Appendix 1), prothrombin time (PT)/international normalized ratio (INR) (as a proxy for routine coagulation tests) and add-ons for the preimplementation period and 4 subsequent implementation time periods:

Before implementation: July 1–31, 2017
After 8000 implementation: October 1–31, 2017
After 8100 implementation: February 1–28, 2018
After coagulation testing: July 1–31, 2018
Stabilization: June 1–30, 2019 for chemistry and PT/INR TAT analysis; March 1–March 31, 2019 for add-on TAT analysis

The TATs (median and interquartile range [IQR]) from specimen receipt to test resulting were calculated each day throughout all 5 time periods for inpatient and emergency department (ED) orders. We also calculated the average, median, and 90th-percentile percentage decrease in TAT for all periods relative to preimplementation. TAT (median and IQR) for add-on tests was calculated from the time an add-on request was received in the laboratory to test resulting. We performed the evaluation of the statistical significance of the differences in TAT using the Wilcoxon rank-sum test.

Results

Implementation and Workflow Analysis

The 10-phase implementation began in January 2017 and ended in May 2018. The numerous operational challenges presented by this process are multiphase and complex (**Table 1**), which mandated careful planning and execution by our multidisciplinary team. Before installation, we ensured the necessary renovations of the specimen-processing area were complete and the blood gas analyzers were moved to their new location, to free up space for additional instrumentation. Because we utilized the existing laboratory space throughout the entire project, we gave particular attention to minimizing operational disruptions that may result from frequent movements of large pieces of instrumentation, as well as preserving TAT.

The Cobas 6000 analytical line remained fully operational while the new systems were being installed and validated. During stages 4, 5, and 6, the Cobas 6000 was replaced with the Cobas 8000 line (**Figure 1A**), while maintaining our full test menu and keeping up with our TAT standards. We strived to maintain 1 preanalytical line (MPA or 8100) fully operational throughout most implementation periods (**Figure 1B**).

We also worked closely with the engineering department at the hospital to find adequate space for power, electrical, and heating, ventilation, and air conditioning (HVAC) requirements and to install temporary power and data drops for the validations and implementation. Our internet technology (IT) team was instrumental in helping us overcome numerous IT challenges (**Table 1**), such as building and testing the interface and the middleware rules for the new system, reprogramming our old MPA line, and maintaining a working interface for the Cobas 6000 and 8000 lines while using different middleware platforms.

Impact on TAT

The TAT for tests received from the ED (**Figure 2A**) and from inpatients (**Figure 2B**) decreased significantly for all 4 implementation periods, compared with the preimplementation period (P <.005). The TAT variability also decreased for the ED and inpatient tests (**Figure 2**).

Compared with the preimplementation period, the percentage reduction in median TAT for the ED was 14% after implementation of the 8000 analyzer, 20% after the 8100 analyzer, 20% after the coagulation testing, and 25% for the stabilization time period. The corresponding percentage reduction in median TAT for inpatient locations was 13% after the 8000 was implemented, 27% after the 8100 was implementd, 25% after the coagulation testing, and 27% for the stabilization time period. Compared with the

Lab Medicine 2021;52;329–337 331
DOI: 10.1093/labmed/lmaa108

Table 1. Phased Implementation Using Existing Laboratory Space (January 2017–May 2018)[a]

Stage/ Implementation Phase	Time Required	Goals	Operational Challenges	Information System Challenges
Stage 1 (preimplementation)	6 months	Renovate the processing area and move blood gas to create space	Maintaining TAT and validating blood gas analyzers after the move	Installation of new hardware and blood gas interface
Stage 2 (preimplementation)	3 months	Move coagulation analyzers and install and validate new Cobas 8000 lines, while maintaining MPA connection to Cobas 6000	Installing a reliable water and HVAC system	Building and testing interface and middleware rules for new analyzers; installing temporary power and data drops for instruments
Stage 3 (preimplementation)	1 week	Disconnect MPA from Cobas 6000	Increase TAT due to manual processing (continued to use MPA to aliquot)	Reprogramming the MPA to meet operational needs
Stage 4 (preimplementation)	2 weeks	Remove 1 Cobas 6000 line and replace with a new Cobas 8000 line	Ensuring analytes were included on at least 1 line and there was redundancy for critical analytes	Maintaining interfaces with 6000 and 8000 lines using different middleware
Stage 5 (preimplementation)	2 weeks	Remove second Cobas 6000 line and replace with second Cobas 8000 line	Same as stage 4	Same as stage 4
Stage 6 (preimplementation)	1 week	Remove last Cobas 6000 line and operate on 2 Cobas 8000 lines	Ensure all analytes are included and that there is redundancy on the 2 lines	NA
Stage 7 (after implementation of the 8000 line)	1 week	Go live with all 3 Cobas 8000 lines and move MPA to make room for the 8100 line	Manual processing during MPA move	Setting up MPA after the move; installing temporary power and data drops
Stage 8 (after implementation of the 8100 analyzer)	2 months	Installing and validating the 8100 line	Maintain processing TAT while programming the 8100 line and training staff	Programming the 8100 line (eg, sorting, aliquoting, specimen routing, priority status, and archiving)
Stage 9 (after coagulation analyzers were implemented)	1 month	Connect coagulation analyzers	Delay due to need to upgrade the analyzers to ensure connectivity	Programming (eg, connections and routing)
Stage 10 (after implementation of the 8100 line)	1 week	Remove MPA	This stage occurred before stage 9 due to delays	NA

TAT, turnaround time; MPA, modular preanalytical (the original Cobas Automated Processing line used); HVAC, heating, ventilation, and air conditioning; NA, nonapplicable.
[a]All Cobas lines mentioned herein are manufactured by F. Hoffman-La Roche Ltd.

preimplementation period (n = 61,990), the ED test volumes increased to 62,620 after the implementation of the 8000 line, 64,204 for the stabilization period, and 66,061 for the poststabilization time period.

The corresponding volumes for inpatient locations were 300,142 for the preimplementation period, 303,585 after the 8000 analyzer was implemented, 319,754 during stabilization, and 333,483 after stabilization. There was a modest decrease in testing volume for ED patients (n = 56,095) and inpatients (n = 275,084) for the period after implementation of the 8100, relative to the previous, post–8000-analyzer-implementation period.

The median TAT for PT/INR decreased from 29 minutes (90% confidence interval [CI] = +/–0.60) during the preimplementation period to 26 minutes (90% CI = +/–0.54) after the 8000 analyzer was implemented, 27 minutes (90%

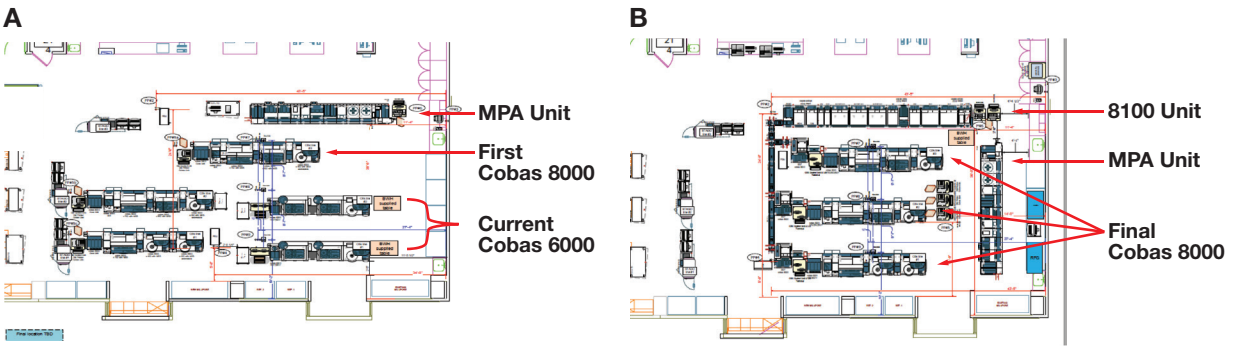

Figure 1

Stages 4 and 9 of implementation. A, Configuration of the laboratory during stage 4 of implementation. The specimen processing area renovations were complete. The new Cobas 8000 units were installed and validated (all Cobas products are manufactured by F. Hoffman-La Roche Ltd). One of the old Cobas 6000 lines was removed and replaced with the first Cobas 8000 unit. The lines were not connected to preanalytical automation (MPA [modular preanalytical; our previous automated-processing line] or 8100) during this stage. B, Configuration of the laboratory during stage 9. The MPA was relocated to accommodate the 8100 line for validation and implementation. The MPA remained operational before and after its relocation, and all 3 Cobas 8000 units were fully operational.

CI = +/–0.50) after the coagulation period, and 25 minutes (90% CI = +/–0.58) at system stabilization (P <.005; data not shown). There was an increase in TAT for the post–8100-analyzer-implementation period to 34 minutes (90% CI, +/–0.64).

Add-on Specimens Analysis

The ED ordered the highest percentage of add-ons during each time period (25.8% before implementation, 20.3% after implementation of the 8000 analyzer, 25.5% after implementation of the 8100 analyzer, 20.7% after the coagulation period, and 21.4% for the stabilization period). The top 10 add-ons during all time periods were procalcitonin, creatine kinase (CK), thyroid-stimulating hormone (TSH), magnesium, lactate dehydrogenase (LDH), troponin T, triglycerides, phosphorus, hepatic function panel, and N-terminal pro-brain natriuretic peptide (NT-proBNP). There was a significant decrease in median TAT and its variability in the period after the 8100 analyzer was implemented (30 minutes; IQR, +/–26), after the coagulation period (30 minutes, IQR, +/–24), and during the stabilization period (28 minutes; IQR, +/– 19) relative to the preimplementation period (66 minutes; IQR +/–48; P <.005; **Figure 3**). The TAT increase noted in the period after the 8000-analyzer implementation (median, 77 minutes; IQR, +/–61) might have occurred because of equipment relocation causing MPA downtime.

The percentage of add-ons requested within 8 hours from original specimen receipt remained consistent across all time periods: 81.4% (9975/12,258) before implementation, 82.4% (11,098/13,466) after implementation of the 8000 analyzer, 82.7% (10,746/12,993) after implementation of the 8100 analyzer, 82.5% (12,169/14,751) after coagulation, and 79.3% (10,364/13,075) for the stabilization time period. The median TAT for specimens retrieved from AOB was 25 minutes (90% CI, +/–1.60 minutes), which is significantly lower than the 53 minutes for specimens not retrieved from AOB (90% CI, +/–1.03 minutes; P <.005). The percentage of add-on requests that the laboratory was unable to fulfill did not change across time periods (9.6% vs 10.8% vs 14.9% vs 6.5% vs 6.0% for the pre-implementation, post-8000, post 8100, post-coagulation implementation, and the stabilization period, respectively).

Discussion

This study examined several important aspects of the introduction of next generation automation in our hospital-based clinical laboratory. Our goals in upgrading our first-generation automated system were to not only replace aging instruments but to also decrease test TAT and variability, increase testing capacity, and improve the workflow.

Lab Medicine 2021;52;329–337
DOI: 10.1093/labmed/lmaa108

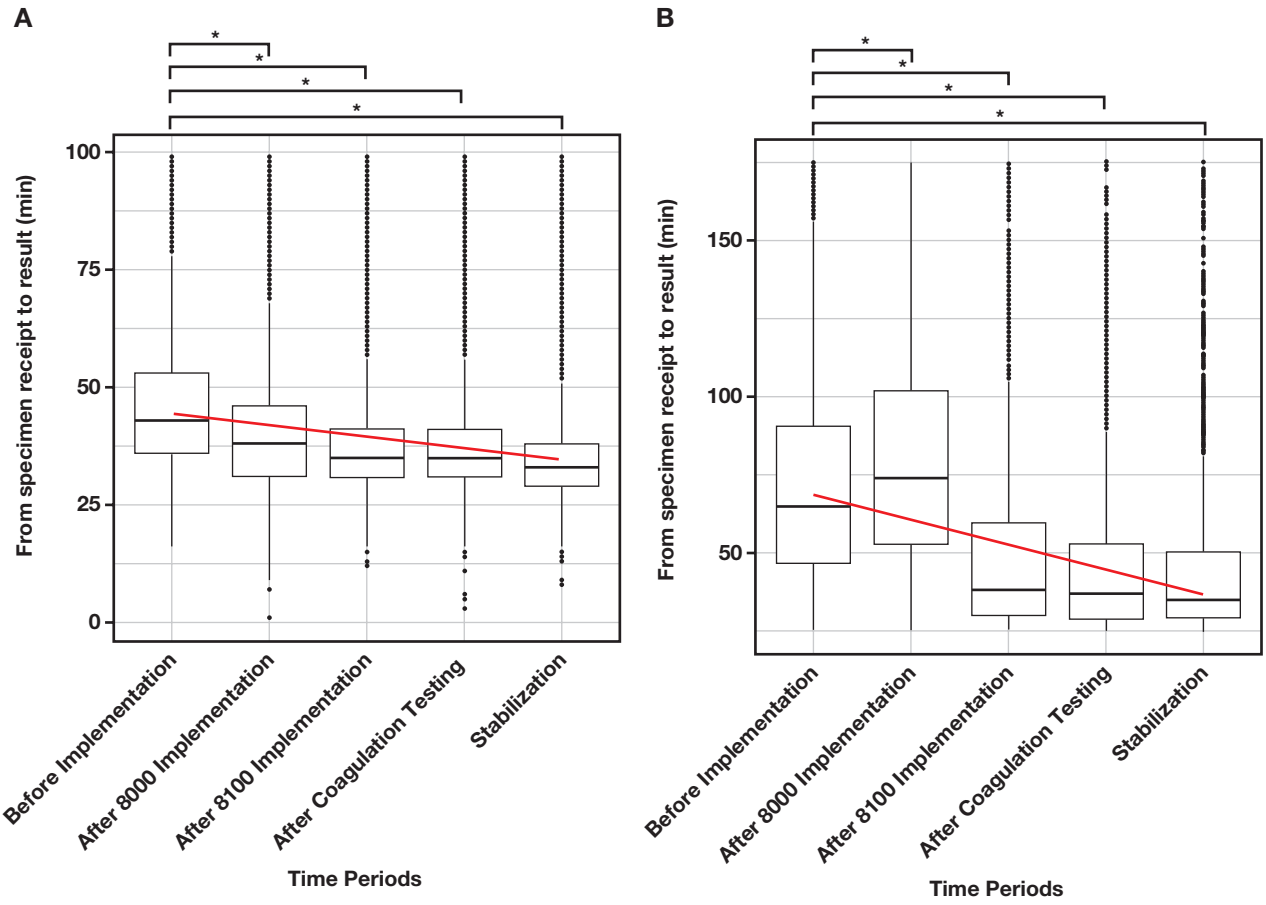

Figure 2

Boxplots (median and interquartile range [IQR]) showing turnaround time (TAT) improvements for each period of implementation. The trendline illustrates the decrease in TAT across time periods. * indicates *P* <.005 for the downward trend, compared with the preimplementation period. A, Emergency department. B, Inpatient settings.

Several features of the new system held the promise of helping us achieve these goals, including increased throughput, the option of adding a third centrifuge, a larger capacity AOB, ability to perform primary-tube specimen collection, and bidirectional connectivity to the chemistry analyzers.

The new automated systems yielded markedly shortened and less variable TAT variability for first-line and add-on chemistry tests. We note that these improvements were sustained, attesting to the robustness of the new system and operational changes. In fact, we experienced a faster TAT after the 8100-analyzer implementation despite the increase in centrifugation time from 5 minutes to 7 minutes.

The impact on coagulation testing, although statistically significant, was not clinically important. We were surprised that we were unable to achieve more significant improvements, despite decreasing the centrifugation time from 10 minutes to 7 minutes and eliminating manual transport of specimens to the analyzers.

We believe this finding was mainly due to 2 challenges we encountered after connecting the 3 STA R Max instruments to the Cobas 8100 track. First, the blue-top tube used (2.7-mL BD vacutainer [Becton, Dickinson and Company]) to collect specimens for PT/INR testing was shorter than other vacutainers placed on the 8100 analyzer, creating the need for a very specific placement of the specimen label on the

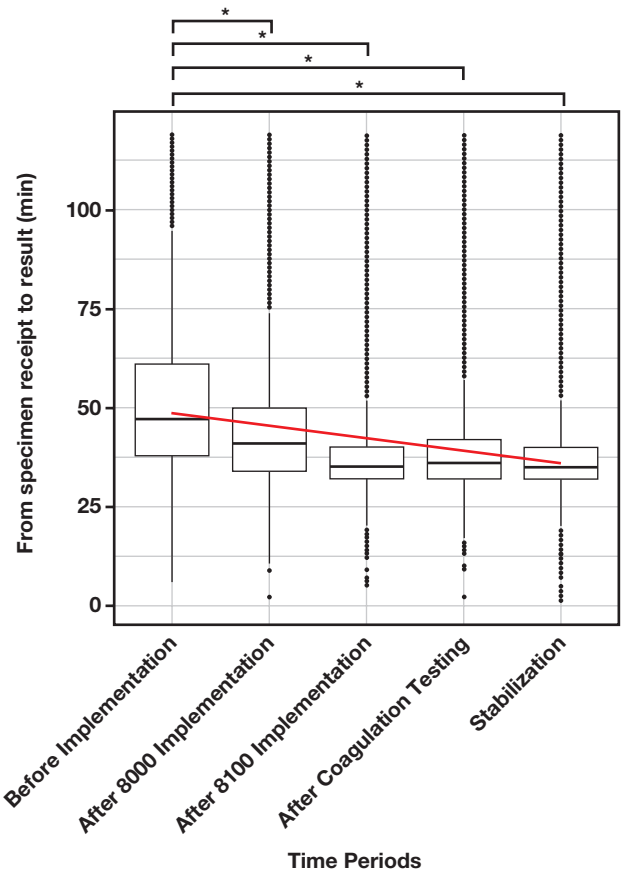

Figure 3

Boxplots (median and interquartile range [IQR]) showing turnaround time (TAT) improvements for add-on tests for each time period. * indicates P <.005.

tube. If the label position on the tube was low, the barcode on the label would be covered by the puck carrying the tube along the Cobas 8100 track. This impacted the barcode readability, triggering the 8100 to route the specimen to the error tray instead of the analyzer for processing. The laboratory began and continues to manually reposition the labels on the tube to prevent these types of errors.

Next, we observed that 40% of PT/INR orders were designated as STAT (for immediate processing) by our providers. The Cobas 8100 was configured to prioritize STAT specimens by allowing the centrifuge to run as soon as the STAT specimen is loaded, instead of waiting for it to be full. This caused the standard 5-position racks to be only partially filled. Since this discovery we have reconfigured the 8100 analyzer to only prioritize specimens from the ED instead all specimens ordered as STAT.

We observed that most of the add-ons were requested and retrieved from the AOB within 8 hours of receipt of the original specimen. Other laboratories with similar add-on patterns should factor this in when deciding whether to purchase costly, large refrigerated modules for longer-term storage. This recommendation should be counterbalanced by considering specimen stability at room temperature if the AOB happens to be the primary site for immediate storage. Our laboratory is writing test-specific rules in our Data Innovations middleware software (Data Innovations Inc., South Burlington, VT) to prevent testing from being performed if the specimen has sat in the AOB beyond the recommended stability window.

There are several aspects of our study that we believe contribute up to and beyond the information available in the literature. We used the existing laboratory space throughout

DOI: 10.1093/labmed/lmaa108

Appendix 1. Tests run on the Cobas 8100 pre-analytical and Cobas 8000 analytical lines

TEST NAMES			
ALBUMIN	CHOLESTEROL	HIGH SENSITIVITY TROPONIN T	PROSTATE SPECIFIC ANTIGEN, TOTAL
ACETAMINOPHEN	CREATINE KINASE MB	IRON	PROSTATE SPECIFIC ANTIGEN,FREE
ALKALINE PHOSPHATASE	CORTISOL	LACTATE	PARATHYROID HORMONE
ALANINE AMINOTRANSFERASE	CREATINE KINASE	LACATATE DEHYDROGENASE	SALICYLATE
AMIKACIN	CREATININE	LIDOCAINE	SODIUM
AMMONIA	DIGOXIN	LIPASE	TRIIODOTHYRONINE (T3)
AMYLASE	DIRECT LDL CHOLESTEROL	LITHIUM	THYROXINE
ASPARTATE AMINOTRASFERASE	ETHANOL	MAGNESIUM	TOBRAMYCIN
VITAMIN B-12	FERRITIN	METHOTREXATE	TOTAL PROTEIN
BILIRUBIN, DIRECT	FOLIC ACID	MYOGLOBIN	TRIGLYCERIDES
BILIRUBIN, TOTAL	FREE THYROXINE (T4)	NT-proB-TYPE NATRIURETIC PEPTIDE	THYROID-STIMULATING HORMONE
BETA-HYDROXYBUTYRATE	GENTAMICIN	PHENOBARBITAL	T UPTAKE
BLOOD UREA NITROGEN	GAMMA GLUTAMYL TRANSFERASE	PHENYTOIN	UNSATURATED IRON BINDING CAPACITY
CALCIUM	GLUCOSE	PHOSPHORUS	URIC ACID
CARBAMAZEPINE	HUMAN CHORIONIC GONADOTROPIN	POTASSIUM	URINE MICROALBUMIN
CARBON DIOXIDE	HDL CHOLESTEROL	PREALBUMIN	VALPROIC ACID
CHLORIDE	HIGH SENSITIVITY C-REACTIVE PROTEIN	PROCALCITONIN	VANCOMYCIN

the process while keeping a preanalytical line and the Cobas 6000 analytical lines fully operational running our full test menu within the standard TAT. Next, instead of focusing on individual tests, we evaluated the TAT for all tests ordered on our automated system. We believe this approach provided a better reflection of the impact of next-generation laboratory automation on the working output of laboratories. Lastly, we have also evaluated the TAT for add-on tests, an important component contributing to the workload in many laboratories.

We plan to apply the lessons learned from the current study to our future planned efforts in microbiology and molecular virology. We hope our study will also help other laboratories in their current and future automation endeavors. **LM**

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DOI: 10.1093/labmed/lmaa108

Verification and Implementation of HIV Antibody Differentiation Testing to Improve Turnaround Time for the HIV Diagnostic Algorithm

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Laboratory Medicine 2021;52:338-345

DOI: 10.1093/labmed/lmaa087

ABSTRACT

Background: Relying on reference laboratories for HIV confirmation testing may lead to delays in treatment and can cause stress for patients who have positive HIV screening results.

Objective: To internalize HIV-1/HIV-2 antibody differentiation testing within the hospital laboratory.

Methods: We analytically verified an HIV antibody differentiation immunoassay and subsequently compared result turnaround times (TATs) for HIV antibody differentiation and HIV-1 qualitative RNA in the months before and after the test internalization.

Results: HIV antibody differentiation was successfully verified. TATs for HIV antibody differentiation and HIV-1 RNA significantly improved, from medians of 40.4 hours and 156.5 hours to medians of 17.7 hours and 56.5 hours, respectively, after the internalization. The 90th-percentile turnaround times declined by 72% and 44%, respectively.

Conclusions: It is feasible for a hospital laboratory to verify HIV antibody-differentiation testing. Its implementation may considerably improve result TATs for the HIV diagnostic algorithm.

Keywords: HIV, turnaround time, quality improvement, infectious disease, method validation, quality metrics

Current laboratory testing recommendations for the diagnosis of HIV infection include screening in serum or plasma with 4th-generation HIV-1/HIV-2 antigen/antibody combination immunoassays.[1] Reactive antigen/antibody combination immunoassay results should be further tested with an HIV-1/HIV-2 antibody differentiation immunoassay, and HIV-1 nucleic acid testing (NAT) should be used to resolve discordant or indeterminate results.

Although 4th-generation antigen/antibody screening tests are widely available on commonly used clinical laboratory immunoassay platforms, there is currently only 1 FDA-approved antibody differentiation test and only 1 FDA-approved HIV-1 NAT commercially available and approved for diagnostic use within the recommended HIV testing algorithm.[2] Thus, it is common for medical centers to perform HIV screening on site but to send confirmatory testing to reference laboratories.[3] Recent College of American Pathologists (CAP) proficiency testing reports (VM-B 2020 and AHIV-A 2020) indicate that although more than 1100 laboratories report screening with an anti-HIV-1/-2 antibody, HIV-1 p24 antigen combination assay, less than one-third of those laboratories report HIV diagnostic testing algorithm results with an antibody differentiation test. Barriers likely include the need to acquire and implement a separate analyzer that is unique to this test.

Confirmatory testing is of utmost importance to distinguish false-positive HIV screening results from true positives, particularly in areas of low HIV prevalence, where positive results are more likely to be false than true.[4] The waiting period between a reactive screening result and the result of confirmatory testing can be stressful for patients. Reactive screening results in women about to give birth may trigger immediate antiretroviral prophylaxis or even cesarean

Abbreviations:

NAT, nucleic acid testing; CAP, College of American Pathologists; TATs, turnaround times; LIS, laboratory information system; EMR, electronic medical record; ED, emergency department.

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section while waiting for confirmatory test results, and there may be legal and social consequences.[5] Further, initiation of antiretroviral therapy is recommended as soon as possible in the setting of acute HIV infection, in which antibody differentiation results may be negative but HIV RNA is detectable.[6]

Prolonged turnaround times (TATs) may cause delays in therapy while waiting for confirmation to be completed. Alternately, if it is deemed too dangerous to wait, antiretroviral therapy may be initiated, along with its possible adverse effects, for patients who turn out to have had false-positive screening results.

Our hospital laboratory has performed 4th-generation HIV-1/-2 antigen/antibody testing since near its onset but has historically sent out the remainder of the HIV diagnostic algorithm tests to reference laboratories. The Bio-Rad Geenius HIV 1/2 Supplemental Assay (Bio-Rad Laboratories, Inc.), which was FDA-approved in 2014 for HIV antibody confirmation, has several advantageous features over its predecessor, the Bio-Rad Multispot, that may facilitate ease of use in the routine hospital laboratory. In contrast to the Multispot assay, the Geenius has automated test interpretation, fewer steps, no specimen dilution requirement, cassette bar coding, and digital image capture, while maintaining similar analytical and clinical performance characteristics.[7] Thus, our laboratory sought to internalize HIV antibody differentiation testing with the Geenius to improve TAT for HIV confirmation results. We predicted that the ability to complete antibody differentiation testing internally and with faster TAT could, in turn, improve TAT for qualitative HIV-1 RNA, if indicated, despite that we did not internalize HIV-1 RNA testing.

To our knowledge, no information has been published regarding the analytical verification of the Geenius in a United States hospital, rather than reference or public health laboratory, setting. Further, the impact of antibody differentiation test internalization on TAT for the HIV diagnostic algorithm has not been described. In this report, we share our analytical verification of HIV antibody differentiation testing performed on the Geenius, compare TATs for HIV confirmatory testing before and after its implementation, and present the results of our survey of health care providers (hereinafter, providers) regarding the clinical impact of our results.

Materials and Methods

Verification Studies of HIV Antibody Differentiation Testing

This study was conducted at a large (600-bed) acute-care public hospital. The overall prevalence of people diagnosed with HIV in the local region is estimated as 506 per 100,000.[8] We acquired 2 Geenius Readers (Bio-Rad Laboratories, Inc.) and analytically verified the Geenius HIV 1/2 Supplemental Assay in the hospital laboratory. The Geenius HIV 1/2 Supplemental Assay provides a qualitative assessment for the confirmation and differentiation of individual antibodies to HIV-1 and HIV-2 in serum—henceforth, we will refer to this assessment as antibody-differentiation testing.

The test consists of a single-use cassette for immunochromatographic testing with an electronic reader, which automatically interprets the resultant banding pattern and reads the test result at 30 minutes from specimen application. Manufacturer-provided control specimens were used for simple and complex precision studies (with 10 negative and 10 positive controls run across both readers). We tested 10 specimens using an additional HIV-1/HIV-2 Verification Panel (ZeptoMetrix Corporation), to challenge the assay with a variety of known results, including HIV Negative (n = 2), HIV-1 Positive (n = 4), HIV-2 Positive (n = 2), and HIV-2 Positive (n = 2) with HIV-1 cross-reactivity. Remnant patient serum specimens (n = 26) with masked HIV status was also used for an accuracy study against the reference laboratory method.

Data Selection for TAT Analysis

The TAT study period included approximately 5.5 months before the Geenius implementation at the hospital laboratory (preimplementation period, January 1, 2019–June 17, 2019), during which time HIV-1/HIV-2 antibody differentiation and qualitative HIV-1 RNA testing were sent to 2 respective reference laboratories. It also included a period of approximately 6.5 months after the implementation (postimplementation period, June 19, 2019–December 31, 2019), during which time HIV-1/HIV-2 antibody differentiation was performed in-house but qualitative HIV-1 RNA testing was still sent out to the reference laboratory (**Table 1**). All consecutive laboratory results available in the

DOI: 10.1093/labmed/lmaa087

Table 1. HIV Diagnostic Algorithm Testing Workflow Before and After Internalizing the HIV-1/-2 Antibody Differentiation Testing

HIV Testing Algorithm Step	Preimplementation Phase	Postimplementation Phase
4th-generation HIV-1/-2 antigen/antibody combination immunoassay screen	Performed in-house	Performed in-house (no change)
HIV-1/HIV-2 antibody differentiation immunoassay	Specimen sent from hospital laboratory to be performed at reference laboratory 1	Performed in-house (new)
HIV-1 nucleic acid testing	Specimen sent from reference laboratory 1 to be performed at reference laboratory 2	Specimen sent directly from hospital laboratory to be performed at reference laboratory 2

laboratory information system (LIS) for HIV-1/HIV-2 antibody differentiation and diagnostic qualitative HIV-1 RNA testing within the study period were included in the TAT analysis.

Laboratory Methods and Algorithm Workflow

During the study period, 4th-generation HIV-1/-2 antigen/antibody screening of fresh serum specimens was performed with the Roche Elecsys HIV combi assay on 1 of 2 Roche cobas e 602 immunoassay analyzers (both F. Hoffman-La Roche, Ltd.) equipped on an automation line in operation 24 hours a day and 7 days a week. Specimens that were repeatedly reactive were reflexed to HIV-1/HIV-2 antibody differentiation testing performed by the Geenius at the reference laboratory (preimplementation phase) or within the hospital laboratory (postimplementation phase).

Once antibody differentiation testing was implemented in-house, it was performed in daily batched runs, with a consistent daily afternoon cutoff time for specimens to be included in the batch. Results were reported in alignment with terminology recommendations from the American Association of Public Health Laboratories.[2] Specimens that were subjected to antibody differentiation testing and that did not yield a confirmed HIV-1 positive result were further reflexed to qualitative HIV-1 RNA testing by transcription-mediated amplification (Aptima, Hologic, Inc.), which was consistently performed at the reference laboratory throughout the entire study period. HIV-2 NAT was not part of the autoreflex algorithm but was available to providers on request at a reference laboratory; however, it was not ordered during the study period.

TAT Calculations

TAT data was collated from LIS reporting tools (Cerner Corporation). Data stemmed from 1 laboratory orderable and 1 serum specimen per patient for the 4th-generation HIV-1/-2 antigen/antibody screening test. Confirmatory testing was added in the LIS by the laboratory as indicated per the CDC-recommended testing algorithm and internal laboratory policy.[1] TAT was defined as the difference between the time that the specimen was scanned, as received in the hospital laboratory for the screening test and the time that the screening or confirmatory test result was verified in the LIS and thus was available for viewing in the electronic medical record (EMR) by the ordering provider.

Data Analysis

Method verification accuracy data for the Geenius assay was analyzed in EP Evaluator, using the Semi-Quantitative Method Comparison module to assess positive and negative agreement and Cohen Kappa. We conducted TAT analysis and statistical analysis in Microsoft Excel Version 2008 (Build 13127.20408) (Microsoft Corporation). TATs were compared by calculating monthly averages, SDs, medians, and the 90th-percentile TAT, defined as the time (in hours) below which 90% of all results were posted.[9] Two-sample *F*-tests for variances and 2-sample *t*-tests assuming unequal variances were used.

Provider Survey to Assess Clinical Impact

To assess provider satisfaction and clinical impact of the test internalization, a voluntary electronic survey was distributed to ordering providers. The survey link was sent by email to a total of 30 providers, including the top 20 most frequent ordering providers associated with HIV screening orders, based on an order system audit for the study time period and 10 additional attending providers with prominent roles in the emergency department (ED), infectious disease services, and HIV clinic services. Three multiple-choice questions were included within the survey, to assess whether the new test internalization improved the ability to distinguish between false-positive and true-positive results more quickly, the ability to diagnose acute HIV cases more quickly, and the ability to administer necessary HIV

treatment for newly diagnosed patients more quickly (answer choices were "yes", "no", and "uncertain"). Also, a fourth, free-text question was included to request any comments regarding how the test internalization has impacted provider practice and patient care. Survey responses were collated anonymously and were analyzed using Microsoft Forms (Microsoft Corporation).

Results

Method Verification of the Bio-Rad Geenius HIV-1/HIV-2 Supplemental Assay in the Hospital Laboratory

We analyzed 10 negative controls and 10 positive controls from the manufacturer and 10 specimens from a third-party HIV-1/HIV-2 test verification panel on both Geenius Readers. All control results were obtained as intended by the respective manufacturers. The verification panel challenged the assay with 4 intended-result categories, including HIV Negative, HIV-1 Positive, HIV-2 Positive, and HIV-2 Positive with HIV-1 cross-reactivity. The results intended by the verification-panel manufacturer were obtained with 100% concordance across both readers. Subsequently, 26 remnant patient serum specimens with masked HIV status were split, to compare results from our Geenius Readers against results from the reference laboratory. The reference laboratory also used the Geenius for HIV antibody differentiation testing, representing the method our hospital was using for clinical testing before internalizing the Geenius.

Also, 4 specimens from the HIV-1/HIV-2 test verification panel (intended as 2 HIV-2 Positive and 2 HIV-2 Positive with HIV-1 cross-reactivity) were anonymized and mixed in with the remnant patient specimens to include in the accuracy study. These controls were included because of the low likelihood of finding HIV-2 specimens in our remnant patient-serum supply. Results were 100% concordant across the in-house and reference laboratory analysis (Cohen Kappa = 100.0%; **Table 2**).

TAT of HIV Antibody Differentiation Results

TATs of HIV antibody differentiation results from the reference laboratory were averaged monthly for the 5.5 months leading up to the implementation of the Geenius in-house. In this time period, 11,099 total 4th-generation antigen/antibody screening tests were performed, of which 278 (2.5%) were repeatedly reactive and thus, these specimens were sent out for antibody-differentiation testing (**Table 3**). The overall baseline TAT statistics for this time period were a mean of 46.7 hours, SD of 27.6 hours, median of 40.4 hours, and 90th percentile value of 89.3 hours (**Table 4**). Thus, providers and patients could wait an average of approximately 2 days, but possibly as long as 3 to 4 days, to receive results.

TATs were calculated in the same manner for the 6.5 months after the Geenius implementation. In this time period, 14,286 total 4th-generation antigen/antibody screening tests were performed, of which 310 (2.2%) were repeatedly reactive and thus, we performed antibody differentiation tests on them. The overall TAT statistics for this time period were a mean of 15.8 hours, SD of 7.8 hours, median of 17.7 hours, and 90th-percentile value of 24.7 hours. Thus, providers could expect to almost always receive results within 1 day. The 90th-percentile TAT declined by 72% between the pre- and postimplementation periods (**Figure 1**). The TAT decrease between the 2 time periods was significant via *t*-testing (P <.005), and the variance also decreased significantly via *F*-testing (P <.005).

TAT Time of Qualitative HIV-1 RNA Results

TATs of qualitative HIV-1 RNA results from the reference laboratory were averaged monthly for the 5.5 months leading up to the implementation of the Geenius in-house and for the 6.5 months after implementation. The testing location for HIV-1 RNA did not change throughout the study period. However, after implementation, specimens needing HIV-1 RNA testing were sent directly to the testing reference laboratory from the hospital laboratory. From the hospital laboratory, rather than taking an intermediate step to the additional reference-laboratory location, where antibody-differentiation testing had been performed previously.

A total of 57 qualitative HIV-1 RNA tests were performed in the preimplementation period. The overall baseline TAT statistics for this time period were a mean of 161.8 hours, SD of 49.0 hours, median of 156.5 hours, and 90th–percentile value of 222.7 hours (**Table 4**). Thus, providers and patients could wait an average of 6 to 7 days, but possibly as many as 9 to 10 days, to receive results that completed the HIV diagnostic algorithm. TATs were calculated in the same manner for the 6.5 months after the implementation. In this

Lab Medicine 2021;52;338–345 341
DOI: 10.1093/labmed/lmaa087

Table 2. HIV Antibody Differentiation Correlation Study

Test Method (Bio-Rad Geenius by Internal Laboratory)[b]	Reference Method (Bio-Rad Geenius, used by Reference Laboratory)[a]				
	HIV Negative	HIV-1 Positive	HIV-2 Positive	HIV-2 Positive with HIV-1 Cross-reactivity	Total
HIV Negative	7	0	0	0	7
HIV-1 Positive	0	19	0	0	19
HIV-2 Positive	0	0	2[c]	0	2
HIV-2 Positive with HIV-1 Cross-reactivity	0	0	0	2[c]	2
Total	7	19	2	2	30

[a] *The Bio-Rad Geenius is manufactured by Bio-Rad Laboratories, Inc.*
[b] *Specimens were run on 2 Geenius Readers in the internal laboratory, with the same results obtained by each.*
[c] *Manufacturer control specimens known to be HIV-2 positive (n = 2) and HIV-2 positive with HIV-1 cross-reactivity (n = 2) were anonymized and mixed in with 26 unknown patient specimens.*

Table 3. HIV Test Volumes and Positivity Rates During the Study Period

Variable	Preimplementation Phase	Postimplementation Phase
HIV-1/-2 antigen/antibody screen testing volume, no.	11,099	14,286
Screening test results that were repeatedly reactive (requiring HIV antibody-differentiation testing), %	2.5%	2.2%
HIV antibody-differentiation testing volume, no.	278	310
Repeatedly reactive screening test results not confirmed as HIV-1 positive (requiring HIV-1 RNA testing), %	20.5%	20.3%
Total screened specimens requiring HIV-1 RNA testing, %	0.5%	0.4%
HIV-1 RNA testing volume, no.	57	63

Table 4. Turnaround Times for HIV Confirmation Test Results Pre- and Post-implementation of Internalizing HIV Antibody Differentiation Testing

Variable	Average (h)	SD (h)	Median (h)	90th Percentile (h)	Count, No.
Pre-implementation					
HIV antibody differentiation	46.7	27.6	40.4	89.3	278
HIV-1 RNA	161.8	49.0	156.5	222.7	57
False-positive screening results	147.1	38.1	145.0	182.1	44
Acute HIV	200.5	28.7	201.3	232.1	9
Post-implementation					
HIV antibody differentiation	15.8	7.8	17.7	24.7	310
HIV-1 RNA	71.9	48.2	56.5	124.2	63
False-positive screening results	66.3	41.9	56.1	98.9	54
Acute HIV	74.9	34.1	75.5	109.2	6

time period, 63 qualitative HIV-1 RNA tests were performed. The overall TAT statistics for this time period were a mean of 71.9 hours, SD of 48.2 hours, median of 56.5 hours, and 90th percentile of 124.2 hours. Thus, many results were now received within 2 to 3 days. The 90th-percentile TAT declined by 44% between the pre- and postimplementation periods (**Figure 1**). The TAT decrease between the 2 time periods was significant via *t*-testing ($P < .005$); however, the variance did not change significantly via *F*-testing ($P = .45$).

Assessment of Clinical Impact

To further assess the clinical impact of the TAT improvement, we divided the cases into result patterns that were consistent with false-positive HIV antigen/antibody screening results (HIV antigen/antibody screening positive, HIV-1 not confirmed by antibody differentiation, and HIV-1 RNA negative) and result patterns that were consistent with acute HIV (HIV antigen/antibody screening positive, HIV-1

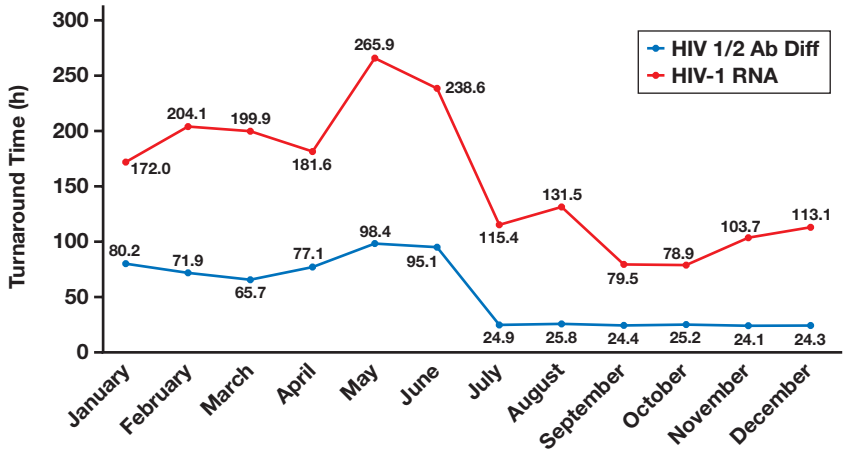

Figure 1

Monthly 90th-percentile turnaround times for 2019, in hours, are shown for HIV-1/HIV-2 antibody differentiation test results (HIV-1/-2 Ab Diff, blue line) and qualitative HIV-1 RNA test results (red line) before and after the hospital laboratory internalized HIV-1/HIV-2 antibody differentiation testing in mid-June.

not confirmed by antibody differentiation, and HIV-1 RNA positive). The TATs for each category were compared before and after implementation (**Table 4**). The 90th-percentile TAT improved by 46% for recognition of false-positive screening results and by 53% for acute HIV diagnosis.

Finally, we surveyed common ordering providers of HIV screening to determine the clinical impact on the test internalization from their perspective. A total of 12 of the 30 providers (40%) who were given a copy of the survey provided responses. Their answers are summarized in **Figure 2**. We discovered that most of the responding providers believed that they were able to distinguish between false-positive and true-positive HIV screening results more quickly (58%), diagnose acute HIV cases more quickly (75%), and administer necessary HIV treatment for newly diagnosed patients more quickly (58%) after the change.

Discussion

Implementation of HIV antibody differentiation testing within the hospital laboratory considerably decreased result TAT for completion of the HIV diagnostic algorithm. The Geenius Reader has a small physical footprint, fits on a benchtop, and is straightforward to validate. Notably, the reagent cost per test for the newly internalized antibody differentiation test is higher than the cost of the same test performed at our reference laboratory, likely owing to high-volume discounts received by the reference laboratory. However, because we chose to batch runs once per day and we have few repeatedly reactive HIV antigen/antibody screens each day, implementation of this testing required minimal full-time equivalent hours of laboratory staff time beyond our status quo. Further, the faster recognition of false-positive screening results may save unnecessary downstream follow-ups and initiation of therapies.

We were pleased with the additional significant improvement of the HIV-1 RNA TAT. We were intrigued that our contracted reference laboratory performed HIV antibody differentiation and qualitative HIV-1 RNA in 2 different laboratory locations. Thus, when specimens needed all 3 steps of the HIV diagnostic algorithm, they had to be transported twice: first from the hospital laboratory to the first reference laboratory for HIV antibody differentiation, and next from the first reference laboratory to the second reference laboratory for HIV-1 RNA. By internalizing HIV antibody differentiation, we could perform the first 2 steps of the algorithm typically within 24 hours and then send the specimen directly to the HIV-1 RNA performing laboratory, if indicated. Other hospitals with more streamlined reference laboratory workflows may not see as significant an improvement as we did.

Reference laboratory TAT can be inconsistent due to variations in specimen transport time, batch schedules

Lab Medicine 2021;52;338–345
DOI: 10.1093/labmed/lmaa087

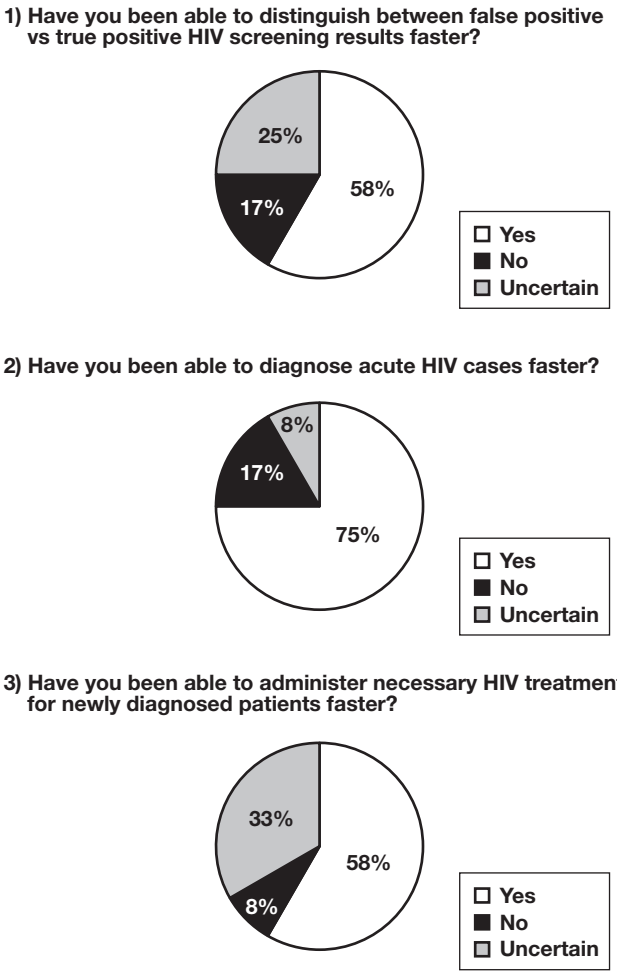

Figure 2

Summary of responses from 12 health care providers to our clinical impact survey. Providers were asked to answer each of the 3 questions in the context of comparing clinical workflow before and after internalization of HIV-1/HIV-2 antibody differentiation testing.

(eg, performing tests on certain days of the week), and reference laboratories often being closed on weekends. This inconsistency was reflected in our data and variances, even after implementation for HIV-1 RNA testing, which was still sent to the reference laboratory. Test internalization and a consistent daily batch schedule yielded a significant decrease in variance and highly predictable TATs for HIV-antibody differentiation, such that we could reliably inform our ordering providers of what to expect. Further, situations requiring rapid confirmation of a positive screening result (eg, obstetrics) off the scheduled testing cycle could be considered on a case-by-case basis.

A limitation to our method verification study was that there was no other standard by which to compare results for antibody differentiation testing, given that the Geenius is the only method that is currently FDA approved and that we were not aware of any laboratories using predecessor to the Bio-Rad Geenius, called the MultiSpot (now discontinued). Thus, we were only able to perform a patient-specimen correlation study with another laboratory using the same analyzer. Recently, the results of a large multicenter study[10] found that the Geenius performed similarly to HIV-1 Western Blot.

Another limitation of the method-verification study was the inability to challenge the assay with all 10 result options of

the Geenius, such as the HIV-1 and HIV-2 Indeterminate Result categories. This limitation occurred due to a lack of vendor or third-party controls available with these intended results, as well as our inability to locate patient specimens known to have these results.

We note that our TAT analysis was specifically focused on assessing the laboratory-controlled timeline of specimen receipt to result verification in the LIS because our analysis included HIV screen orders from various inpatient and outpatient (onsite and offsite) locations. We did not include analysis of TATs using time stamps for order placement or specimen collection, although these timelines may interest others on the clinical side for location-focused quality-assurance investigations.

We also did not factor in the time to result of the initial antigen/antibody screening test in the TAT timelines, although this test was always performed first for every specimen we analyzed in this study. TAT for the antigen/antibody screening step at our hospital is advertised as 2 hours for STAT orders and 4 hours for routine orders. During the study period, the 90th-percentile TAT for all orders was consistently within 2 hours according to internal laboratory quality-assurance monitoring reviewed at monthly intervals. Thus, we did not anticipate that correcting for or subtracting out this part of the TAT would impact the results of our study. Despite this, we acknowledge that rare downtime of the automation line or automated immunoassay analyzers could potentially play a role in delayed TAT for the HIV testing algorithm for some specimens.

Provider feedback regarding the test internalization was overwhelmingly positive. Anonymous free text responses obtained via the provider survey included comments such as "able to same day link to treatment," "early detection and immediate access to care," "reduce reinfection of others," and "there is a real stigma still to receiving an HIV diagnosis, and the importance of removing 'false positive' diagnoses as quickly and accurately as possible cannot be overstated." One comment noted that the timeline was greatly improved but was still longer than a typical patient encounter in the ED, noting "we make the decision on whether to treat for acute infection on clinical grounds, but the rapid TAT helps our HIV medicine colleagues adjust rapidly to the true diagnosis."

In summary, it can be feasible for a hospital laboratory to analytically verify HIV antibody differentiation testing, and its internalization can considerably decrease result TAT for the HIV diagnostic algorithm as a whole. Improvement of TAT for the HIV diagnostic algorithm has many potential positive clinical implications, including the ability to rapidly distinguish false-positive screening results and decreasing the time to diagnose acute HIV infection. **LM**

Acknowledgments

We thank Ruey-Fen Hwang, CLS (ASCP), and Margarita Montano, CLS (ASCP), for their assistance with data acquisition.

Personal or Professional Conflicts of Interest

None declared.

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Trimethylamine N-Oxide is Associated with Heart Failure Risk in Patients with Preserved Ejection Fraction

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Laboratory Medicine 2021;52:346-351

DOI: 10.1093/labmed/lmaa075

ABSTRACT

Background: Trimethylamine N-oxide (TMAO) has been considered to be an independent risk factor of heart failure (HF).

Objectives: To further determine the plasma levels of TMAO in patients who have HF with preserved ejection fraction (HFpEF), and to analyze the relationship between TMAO and HFpEF risk.

Methods: A total of 57 control participants and 61 patients with HFpEF were recruited. We measured and analyzed plasma levels of TMAO and performed biochemical examination of all patients.

Results: The mean (SD) plasma levels of TMAO in patients with HFpEF (6.84 [1.12] μmol/L) were significantly higher than in controls (1.63 [0.08] μmol/L; P <.01). The area under the curve (AUC) of TMAO and N-terminal pro b-type natriuretic peptide (NT-proBNP) was 0.817 and 0.924, respectively, which were determined by receiver operating characteristic (ROC) analysis. TMAO was an independent risk factor in patients with HFpEF, as revealed by univariate and multivariate logistic regression analysis. The level of TMAO was correlated with blood urea nitrogen (BUN), creatinine, and NT-proBNP.

Conclusions: TMAO level was highly associated with HFpEF risk.

Keywords: gut microbiota, trimethylamine N-oxide, heart failure, preserved ejection fraction, risk factors, N-terminal pro b-type natriuretic peptide

Heart failure (HF) is the end stage for most types of cardiovascular diseases. Some patients with HF have *preserved left ventricular ejection fraction* (LVEF), which is defined as HF with preserved ejection fraction (HFpEF). One of the classifications of HF is made by determining the LVEF. If the LVEF is greater than 50%, HF can be defined as HFpEF. When LVEF is less than 40%, HF can be defined as HF with reduced ejection fraction (HFrEF).[1] According to the results of 2 studies,[2,3] patients with HFpEF have diastolic dysfunction. Myocardial remodeling and cardiac fibrosis are known to play important roles in diastolic dysfunction.

Trimethylamine N-oxide (TMAO) is a nutrient metabolite produced by gut microbiota.[4–6] Many nutrient precursors can be metabolized to TMAO, including choline, phosphatidylcholine, and L-carnitine, all of which are derived mainly from red meat, egg yolks, dairy products, and seafood. The results of multiple studies[7–11] have revealed an association between TMAO level and cardiovascular risk. These findings have demonstated that TMAO had an association with risk of major adverse cardiac events. The results of a previous study by some of us[12] indicated that plasma level of TMAO was associated with coronary heart disease (CHD) risk.

Until now, the association between TMAO levels and HFpEF risk has not yet been studied, to our knowledge. Thus, we aimed to assess the relationship between TMAO levels and HFpEF risk. In the present study, we determined the plasma

Abbreviations:

HF, heart failure; LVEF, left ventricular ejection fraction; HFpEF, HF with preserved ejection fraction; HFrEF, HF with reduced ejection fraction; TMAO, trimethylamine N-oxide; CHD, coronary heart disease; BUN, blood urea nitrogen; NT-proBNP, N-terminal pro b-type natriuretic peptide; ALT, alanine transaminase; TC, total cholesterol; TG, triglycerides; LC-MS/MS, liquid chromatography with tandem mass spectrometry; HPLC, high-performance liquid chromatography; IQR, interquartile; ROC, receiver operating characteristic; AOC, area under the curve; CI, confidence interval; OR, odds ratio

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levels of TMAO in patients with or without HFpEF. The association between TMAO levels and HFpEF risk was evaluated using ROC, as well as univariate and multivariate logistic regression analysis.

Material and Methods

Participants

Between May, 2016, and August, 2017, we enrolled 166 patients with or without HFpEF from the First Affiliated Hospital of the Harbin Medical University, Harbin, China, in our study. The inclusion criterium for the HFpEF group was having chronic HFpEF, as diagnosed by a cardiologist who specializes in HFpEF. Patients with LVEF of less than 40%, severe hepatic disease, simultaneous infection, and/or renal dysfunction diseases were excluded.

We recruited a total of 88 control participants and 78 patients with HFpEF for this study. To establish more rational comparisons between participants, we identified 31 controls and 17 patients with HFpEF who did not have matched clinical or demographic characteristics. Therefore, we reserved plasma specimens from 57 controls and 61 patients with HFpEF for detailed statistical analysis. The procedures for handling plasma specimens and the study protocols were approved by the Institutional Ethics Committees of the First Affiliated Hospital of Harbin Medical University.

Demographics and Laboratory Examinations

The participants who enrolled had undergone physical examination and were assessed by a cardiologist. Echocardiography was conducted by a cardiac sonographer who was masked to the results of this study. Also, we collected information regarding the demographic and clinical characteristics of participants, along with any HFpEF-related symptoms. Serum levels of albumin, hemoglobin, blood urea nitrogen (BUN), creatinine, N-terminal pro b-type natriuretic peptide (NT-proBNP), alanine transaminase (ALT), total cholesterol (TC), and triglycerides (TG) were tested in the clinical laboratory of the First Affiliated Hospital of Harbin Medical University.

Specimen Collection and Quantification of Plasma TMAO Levels

TMAO was collected from the plasma of each participant and determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS), as described previously.[12] Briefly, 5 μL of each plasma specimen after treatment was injected into a Luna Silica column (Phenomenex, Inc.) and was separated by a high-performance liquid chromatography (HPLC) system (Agilent 1100; Agilent Technologies, Inc.) at 24°C. Then, we determined the TMAO of the plasma

Table 1. Baseline Characteristics of the Patients

Characteristic	Group		*P* Value
	Control (*n* = 57)	HFpEF (*n* = 61)	
Age (y), mean (SD)	61.91 (9.58)	63.23 (13.83)	.55
Male, no. (%)	23 (40.3%)	25 (41.0%)	.94
LVEF (%), mean (SD)	63.75 (6.69)	56.99 (8.48)	<.01
LAD (mm), mean (SD)	35.55 (5.03)	40.26 (5.97)	<.01
LVEDD (mm), mean (SD)	46.50 (7.16)	49.68 (7.24)	.02
Albumin (g/L), mean (SD)	41.81 (4.67)	36.87 (4.36)	<.01
Hemoglobin (g/L), mean (SD)	138.62 (16.77)	123.54 (22.99)	<.01
BUN (mmol/L), mean (SD)	5.51 (1.55)	7.78 (6.31)	.009
Creatinine (μmol/L), mean (SD)	61.22 (11.57)	79.49 (46.71)	.004
NT-proBNP (pg/mL), median (IQR)	106.3 (41.36–302.45)	2212.5 (1138.00–3291.75)	<.01
ALT (U/L), mean (SD)	19.06 (17.09)	22.27 (12.65)	.26
TC (mmol/L), mean (SD)	4.93 (1.00)	4.46 (1.31)	.03
TG (mmol/L), mean (SD)	1.85 (1.17)	1.74 (1.12)	.61
TMAO (μmol/L), mean (SD)	1.63 (0.08)	6.84 (1.12)	<.01

HFpEF, heart failure with preserved ejection fraction; LVEF, left ventricular ejection fraction; LAD, left atrial diameter; LVEDD, left ventricular end-diastolic dimension; BUN, blood urea nitrogen; IQR, interquartile range; NT-proBNP, N-terminal pro-brain type natriuretic peptide; ALT, alanine aminotransferase; TC, total cholesterol; TG, triglycerides; TMAO. trimethylamine N-oxide.

Lab Medicine 2021;52;346–351
DOI: 10.1093/labmed/lmaa075

specimen by using a triple quadrupole mass spectrometer (API 4000; Dh Tech. Dev. Pte. Ltd.).

Data Analysis and Statistics

Data from continuous variables that fit normal distribution were shown as mean (SD) and were analyzed using an independent-samples *t* test. Data that fit non-normal distribution were shown as median (interquartile [IQR] range) and were analyzed using the Mann-Whitney U test. Categorical variables were shown as number (%) and were analyzed using χ^2 testing. The receiver operating characteristic (ROC) curve was used to assess HFpEF risk. Univariate and multivariate logistic regression analysis were applied to evaluate the association between the TMAO and HFpEF risks. The correlation of TMAO with risk factors of HFpEF was measured using Pearson analysis. All statistical analyses were performed with SPSS software, version 20.0 (IBM Corporation). Statistical significance was accepted at $P < .05$ for all analyses.

Results

Patient Characteristics and Plasma TMAO Levels in Patients with HFpEF

This study consisted of 57 controls and 61 patients with HFpEF (**Table 1**). TMAO was separated from control and HFpEF plasma specimens. Plasma levels of TMAO were determined using the LC-MS/MS method. The plasma level of TMAO was 6.84 (1.12) μmol per L in the HFpEF group and 1.63 (0.08) μmol per L in the control group ($P < .01$; **Figure 1A**). The plasma levels of NT-proBNP were higher in patients with HFpEF than in controls ($P < .01$; **Figure 1B**). These results indicated that TMAO and NT-proBNP were shown to have a significant association with HFpEF ($P < .01$).

ROC Analysis for the Association between TMAO and HFpEF

To determine the association between TMAO and HFpEF, ROC curves were constructed for TMAO or NT-proBNP based on plasma concentration. The results of area under the curve (AUC) analysis indicated that TMAO (0.817; 95% confidence interval [CI], 0.732–0.901) and NT-proBNP (0.924; 0.867–0.981) represented significant values (**Figures 2A** and **2B**). These results indicated that TMAO and NT-proBNP were associated with HFpEF risk. When

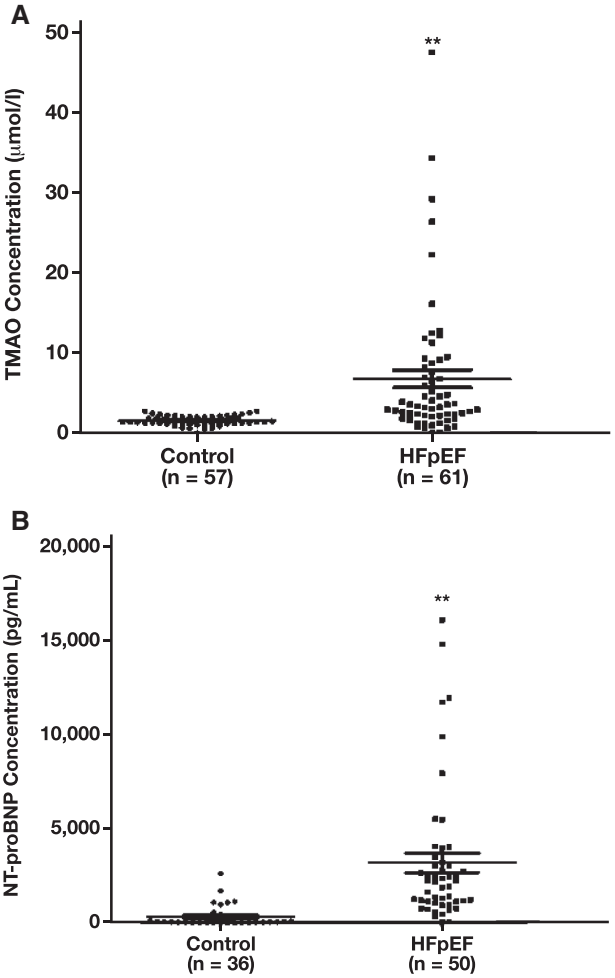

Figure 1

The plasma levels of trimethylamine N-oxide (TMAO) and N-terminal pro b-type natriuretic peptide (NT-proBNP) in the control group and the group with heart failure with preserved ejection fraction (HFpEF). A, Levels of TMAO in the control and HFpEF groups. B, Levels of NT-proBNP in the control and HFpEF groups. ** indicates $P < .01$, vs the control group.

combining TMAO with NT-proBNP, an AUC value of 0.937 (95% CI, 0.884–0.990) was obtained, which was similar with NT-proBNP only (**Figure 2C**).

Logistic Regression Analysis for the Association between TMAO and HFpEF

The results of univariate analysis with logistic regression showed that the odds ratio (OR) was 2.799 (95% CI, 1.682–4.660) for TMAO and 1.002 (1.001–1.003) for NT-proBNP between the control and HFpEF groups, respectively (**Table 2**). The multivariate logistic regression analysis revealed that the OR values

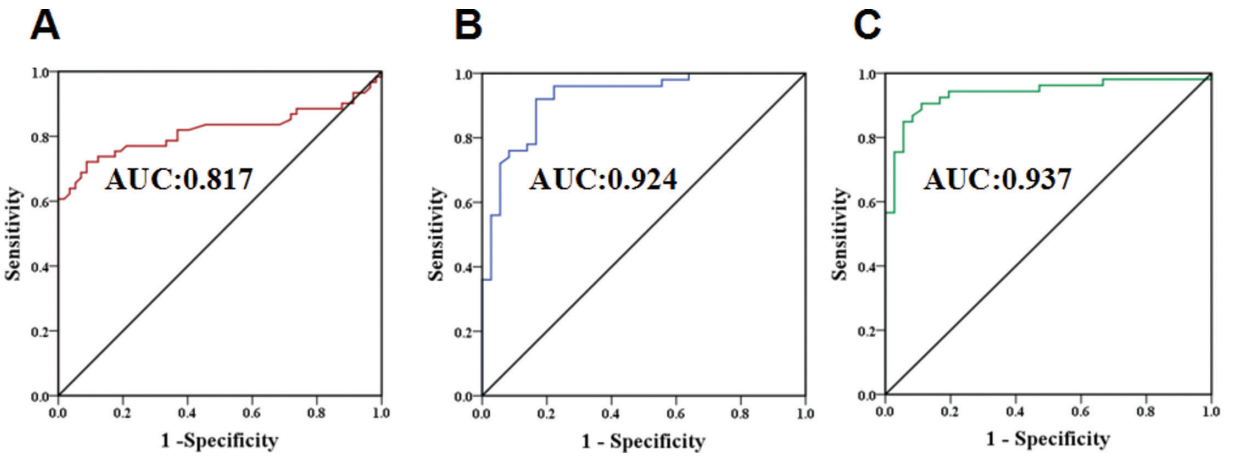

Figure 2

Receiver operating characteristic (ROC) curves of the diagnosis ability of trimethylamine N-oxide (TMAO) and N-terminal pro b-type natriuretic peptide (NT-proBNP) for heart failure with preserved ejection fraction (HFpEF). The area under the ROC curve was determined to evaluate the predictive power of circulating TMAO (A), NT-proBNP (B), and TMAO + NT-proBNP (C) for HFpEF.

Table 2. Univariate Logistic Regression Analysis for Risk Factors of HFpEF

Characteristic	OR	95% CI	*P* Value
Age	1.009	0.979–1.041	.55
Male sex	1.027	0.492–2.141	.94
LVEF	0.895	0.850–0.943	<.01
LAD	1.188	1.089–1.296	<.01
LVEDD	1.066	1.009–1.127	.02
Albumin	0.765	0.680–0.860	<.01
Hemoglobin	0.960	0.938–0.982	<.01
BUN	1.277	1.070–1.525	.007
Creatinine	1.029	1.007–1.051	.01
NT-proBNP	1.002	1.001–1.003	<.01
TMAO	2.799	1.682–4.660	<.01

HFpEF, heart failure with preserved ejection fraction; OR, odds ratio; CI, confidence interval; LVEF, left ventricular ejection fraction; LAD, left atrial diameter; LVEDD, left ventricular end-diastolic dimension; BUN, blood urea nitrogen; NT-proBNP, N-terminal pro-brain type natriuretic peptide; TMAO. trimethylamine N-oxide.

Table 3. Multivariate Logistic Regression Analysis for Risk Factors of HFpEF

Characteristic	OR	95% CI	*P* Value
LVEF	0.953	0.837–1.084	.46
LAD	0.871	0.725–1.046	.14
LVEDD	1.153	0.950–1.401	.15
Albumin	0.747	0.583–0.957	.02
Hemoglobin	1.020	0.957–1.087	.54
BUN	0.831	0.478–1.446	.51
Creatinine	0.984	0.901–1.074	.71
NT-proBNP	1.002	1.001–1.003	.005
TMAO	3.199	1.071–9.553	.04

HFpEF, heart failure with preserved ejection fraction; OR, odds ratio; CI, confidence interval; LVEF, left ventricular ejection fraction; LAD, left atrial diameter; LVEDD, left ventricular end-diastolic dimension; BUN, blood urea nitrogen; NT-proBNP, N-terminal pro-brain type natriuretic peptide; TMAO, trimethylamine N-oxide.

were 3.199 (1.071–9.553) for TMAO and 1.002 (1.001–1.003) for NT-proBNP between the control and HFpEF groups, respectively (Table 3). These results further indicated that TMAO was an independent risk factor in patients with HFpEF.

Pearson Analysis for Correlations between TMAO and Risk Factors for HFpEF

Pearson correlation analysis was performed to identify possible correlations between TMAO level and other risk factors for HFpEF. The data showed that TMAO is significantly correlated with hemoglobin, BUN, creatinine, and NT-proBNP, (*P* <.01; Table 4). These result indicate that TMAO is significantly correlated with other risk factors of HFpEF.

Discussion

This study investigated the plasma levels of TMAO among patients diagnosed with or without HFpEF. The plasma TMAO levels of the patients with HFpEF were significantly higher than those levels in the controls, and ROC curve

Lab Medicine 2021;52;346–351
DOI: 10.1093/labmed/lmaa075

Table 4. Correlation Analysis for the Association of TMAO with Risk Factors of HFpEF

Risk Factors of HFpEF	TMAO	
	Coefficient	*P* Value
LVEF	0.046	.73
LAD	0.004	.97
LVEDD	−0.004	.97
Albumin	−0.206	.11
Hemoglobin	−0.465	<.01
BUN	0.656	<.01
Creatinine	0.605	<.01
NT-proBNP	0.311	.03

TMAO, trimethylamine N-oxide; HFpEF, heart failure with preserved ejection fraction; LVEF, left ventricular ejection fraction; LAD, left atrial diameter; LVEDD, left ventricular end-diastolic dimension; BUN, blood urea nitrogen; NT-proBNP, N-terminal pro-brain type natriuretic peptide.

analysis revealed significant AUC values for TMAO and NT-proBNP. Univariate and multivariate logistic regression analysis revealed that TMAO was an independent risk factor in patients with HFpEF. The level of TMAO was correlated with hemoglobin, BUN, creatinine, and NT-proBNP. TMAO level was highly associated with HFpEF risk.

At present, the findings of some studies[13,14] suggest that the microbial metabolite TMAO is linked to cardiovascular diseases. Accumulating data have also revealed[13,14] an association between plasma levels of TMAO and HF.

The results of a previous study[15] revealed that TMAO was an independent risk factor for HF, which followed higher mortality risk. However, the correlation between plasma TMAO level and HFpEF is unclear. In our study findings, the plasma level of TMAO was 6.84 (1.12) μmol per L in the HFpEF group and 1.63 (0.08) μmol per L in the control group (P <.01; **Figure 1A**). These results showed that TMAO has a significant association with HFpEF. Further, the ROC analysis revealed that the AOC of TMAO was 0.817, which predicts HFpEF. Also, the results of univariate and multivariate logistic regression analysis showed that TMAO was an independent risk factor for HFpEF.

The exact mechanism of HFpEF remains unknown. However, cardiac fibrosis may be an essential pathological factor for the development and progression of HFpEF. When cardiac fibrosis occurs, cardiac fibroblasts transform to myofibroblasts. Extracellular matrix initiate synthesis and release,[16,17] which affect cardiac diastolic function. The exact role of TMAO on HFpEF is unclear. The results of 2 studies[18,19] have determined that dietary intake affects gut microbiome and that TMAO level can induce HF and adverse cardiac events.0

A high plasma level of TMAO is associated with diastolic dysfunction and mortality.[4,15] In an experimental study, animals whose levels of TMAO had been artificially increased by the investigators had significantly enhanced severity of HF and increased incidence of cardiac fibrosis.[20] These data imply that TMAO is a potential therapeutic target for cardiac fibrosis and HFpEF.

Limitations

The present study had the following limitations. First, it is a single-center study with a small study population. It is necessary that more, larger specimen studies are implemented, to more strongly confirm the association of TMAO levels and HFpEF risk. Second, our study was observational and retrospective. Future studies will be required to provide prospective data regarding TMAO and mortality in patients with HFpEF.

Conclusions

In our study findings, TMAO levels were highly associated with HFpHF risk. The importance of our study was that we identified TMAO as a potential biomarker for future diagnoses of HFpHF. LM

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant no. 81400250, 81870169) and the Natural Science Foundation of Heilongjiang Province (grant no. H2017040).

Personal and Professional Conflicts of Interest

None reported.

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DOI: 10.1093/labmed/lmaa075

Stable Plasma Sample Storage in Acetonitrile for Angiotensin and Aldosterone Analysis

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Laboratory Medicine 2021;52:352-356

DOI: 10.1093/labmed/lmaa079

ABSTRACT

Background: Angiotensin I, II (AI, AII) and aldosterone are unstable in plasma specimens at room temperature, making it difficult for collect samples for remote regions in centralized and collaborative studies. Here we introduce a stable storage method which do not require cold conditions..

Methods: Acetonitrile was added to the plasma to 60%, and then the supernatants were kept at 4°C and room temperature for 0, 1, 2, 3, 10 and 30 days. AI, AII and aldosterone were extracted and analyzed by chemiluminescence immunoassays.

Results: AI, AII and aldosterone were well retained in the supernatant under this method. The intra- and inter-day CVs of this method were all below 10%. The levels of AI, AII and aldosterone by this method remained stable for 30 days at room temperature.

Conclusion: Addition of 60% acetonitrile in the plasma provides a stable storage method for clinical AI, AII and aldosterone.

Keywords: angiotensin I, angiotensin II, aldosterone, acetonitrile, immunoassay analyses

The renin-angiotensin-aldosterone system is an important structure that regulates blood pressure, bodily fluids, and electrolyte balance.[1] Overactivation of this system often causes hypertension and other health problems[2-4] and is usually indicated by blood levels of angiotensin I and II (AI, AII) and aldosterone determined by clinical laboratories.

Clinical plasma specimens for angiotensin and aldosterone analysis are often kept at 4°C to minimize their further conversion or degradation. With wide collaboration among the research community and medical laboratories, plasma specimens sometimes must be shipped over a long distance, which may take days, during which this cold temperature is not easily maintained.

AI, AII, and aldosterone are small molecules and can potentially be extracted by organic solvents. Acetonitrile is a common organic compound used for the extraction of small peptides and metabolites in biological experiments,[5,6] and it has been used routinely in peptide extraction for mass spectrometry–based quantitative proteomics.[7-9] Researchers have also used it to preserve proteins by dehydration in polymerized acrylamide gels.[10] Acetonitrile, like acetone, precipitates large proteins including enzymes that catalyze AI, AII, and aldosterone, leaving these small molecules extracted in the supernatant. This process makes it possible to add acetonitrile into a plasma or serum specimen to stably extract and store it.

Based on this concept, in this study we tried to add acetonitrile to plasma specimens and keep them at room temperature for 1 month to see whether the AI, AII, and aldosterone remained stable under this condition. This study may enable the long-term shipping of plasma specimens without needing to maintain cold temperatures for angiotensin and aldosterone analyses for centralized studies or globally collaborative research on the renin–angiotensin–aldosterone system.

Abbreviations:

AI, angiotensin I; AII, angiotensin II; ABEI, amino-butyl-ethyl-isoluminol; FITC, fluorescein-5-isothiocyanate; CV, coefficient of variation.

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Materials and Methods

Plasma Storage in Acetonitrile and Extraction

Acetonitrile was added to the plasma specimen to 60% (4 volumes of plasma + 6 volumes of acetonitrile). This concentration of acetonitrile is sufficient to precipitate large protein levels in plasma. A higher concentration may increase the chances of angiotensin peptide and aldosterone loss. After centrifuge at 20,000*g* for 5 minutes, the supernatant was taken and stored at -80°C immediately as Day 0, or kept at 4°C or at room temperature for 1, 2, 3, 10, and 30 days. To analyze AI, AII, and aldosterone, the supernatant specimens were dried in a vacuum concentrator (SpeedVac, Thermo Scientific) and then resuspended with deionized water at a volume equal to that of the plasma (40% of the total taken supernatant) before measurements by immunoassay.

AI, AII, and Aldosterone Loss Determination

To determine whether AI, AII, or aldosterone could be efficiently extracted by acetonitrile, ^{125}I-labeled AI, AII, or aldosterone was added to the plasma and then serially diluted 3-fold with the same plasma. Then acetonitrile was added to each of these samples to 60%. After the sample was centrifuged, the supernatant was taken and dried, and resuspended with deionized water for radioactivity measuring in 1 minute. The same plasma specimens that were added to serially diluted ^{125}I-labeled AI, AII, or aldosterone but that received no acetonitrile were used as controls.

Large Pool of Plasma Specimen Collection

Clinical plasma specimens supplemented with 3 mM EDTA, 1.6 mM dimercaptopropanol, and 3.4 mM 8-hydroxyquinoline sulfate were collected in our laboratory. Specimens with an increased white blood cell count, positive hepatitis B surface antigen, or any other signs of infections were excluded. All specimens were negative in human immunodeficiency virus, hepatitis C virus, and syphilis antibody testing. Specimens were combined, and approximately 30 mL were used for this study.

Specimen Preparation and Storage

Half of the plasma was aliquoted (0.4 mL) to 1.5 mL vials that contained 0.6 mL acetonitrile each and mixed well. Three specimens with acetonitrile and 3 without acetonitrile were immediately stored in the freezer at –80°C and were labeled as day 0. The rest of the specimens, with or without acetonitrile, were kept at 4°C or at room temperature and were harvested at days 1, 2, 3, 10, and 30 in the freezer (–80°C) for later analysis.

For the analyses of angiotensin and aldosterone, specimens with acetonitrile were taken out and centrifuged at 20,000*g* for 3 minutes. We collected and dried 0.8 mL supernatant on the SpeedVac Vacuum Concentrator (Thermo Scientific). Next, 320 μL deionized water was added to resuspend the analytes for analysis after centrifuge (20,000*g* × 3 min).

AI, AII, and Aldosterone Immunoassay

All specimens were analyzed on an automated chemiluminescent immunoassay machine (MAGLUMI X8, Snibe Co, Ltd, Shenzhen, China). In the angiotensin immunoassay, AI or AII in the sample to be measured completes with the AI or AII reagent peptide labeled with chemiluminescence (amino-butyl-ethyl-isoluminol [ABEI]) for binding to the polyclonal AI or AII antibody and was conjugated to magnetic microbeads. In the aldosterone immunoassay, the purified aldosterone in the reagent was labeled with fluorescein-5-isothiocyanate (FITC) and the monoclonal aldosterone antibody was labeled with ABEI. Aldosterone in the sample to be measured competes with the ABEI -labled aldosterone from the reagent for binding of the antibody to be captured by magnetic beads. The final complex (ABEI-antibody-aldosterone-FITC) was separated by anti-FITC on magnetic beads for signal development and measurement. Calibration curves for plasma specimens and extracted specimens were separate.

Data Processing and Statistical Analysis

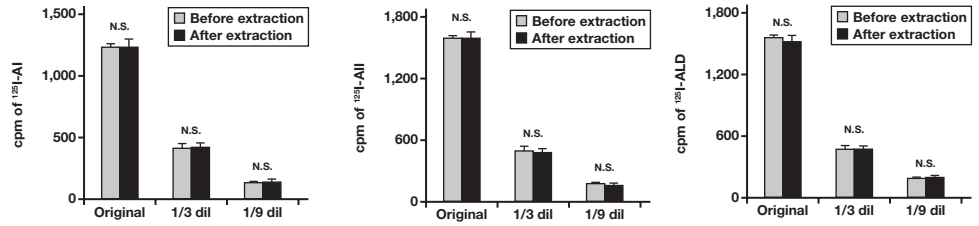
The paired Student's *t* test, 2-way and 1-way analyses of variance, and the Tukey posthoc test were used.

Results

The extraction efficiency results showed that there was no significant difference between the measured radioactivity before and after the acetonitrile extraction; for the original tube with a relatively high level of ^{125}I-labeled AI, AII, or

Lab Medicine 2021;52;352–356
DOI: 10.1093/labmed/lmaa079

A



B

	AI (Angiotensin I, ng/mL)				AI (Angiotensin II, pg/mL)				ALD (Aldosterone, pg/mL)			
	Intraday (n = 4)		Interday (n = 5)		Intraday (n = 4)		Interday (n = 5)		Intraday (n = 4)		Interday (n = 5)	
	SD	CV	SD	CV	SD	CV	SD	CV	SD	CV	SD	CV
Level 1 (low)	0.04	8.17%	0.01	3.36%	2.66	8.56%	0.58	1.87%	7.85	6.11%	5.91	4.60%
Level 1 (high)	0.07	5.93%	0.03	2.56%	7.46	7.52%	1.12	1.12%	11.51	4.47%	13.91	5.44%

Figure 1

Angiotensin and aldosterone extraction by acetonitrile. A, Analyte loss during acetonitrile mediated processing in this methodology. Plasma with serially diluted 125I-labeled AI, AII or ALD were extracted with 60% acetonitrile. The radiation was measured before and after the extraction. cpm, Counts of detected irradiation Per Minute. "Original" indicates the first tube spiked wth 125I-labeled analyte, and "dil" means dilution from the Original tube. B, The precision of the acetonitrile-extraction methodology. Samples were pooled from clinical plasma specimens. Low levels for AI, AII and ADL were 0.43 ng/ml, 31.14 pg/ml, and 128.48 pg/ml respectively; and high levels were 1.13 ng/ml, 99.30 pg/ml, 255.63 pg/ml respectively. Samples were extracted four times a day for five consecutive days. Intra-day: extraction of the same day, and the SD and CV values shown were the average of five from each day (e.g., SD in the table is the average of 5 SD results from each day). Inter-day: SD and CV were calculated from the averages of the five days each. Error bars are the standard deviations. Paired student t tests were used. "N.S." indicates no significant difference.

aldosterone; or for the 3-fold serially diluted tubes with a lower concentration of ^{125}I-labeled analytes (**Figure 1A**). These findings indicated that AI, AII, and aldosterone were almost all retained in the supernatant without significant loss during extraction.

To further show the reliability of this methodology, we repeated this protocol on the same sample 4 times a day for 5 consecutive days. Surprisingly, the intraday and interday coefficients of variation (CVs) were all <10% in the results of all 3 analytes, indicating a very high reproducibility of this methodology (**Figure 1B**).

We therefore investigated whether this acetonitrile storage method could be used to preserve specimens at common temperature conditions. Without acetonitrile in the plasma, although aldosterone remained stable over the 30 days of storage, AI and AII began to increase after 3 days (**Figure 2**). However, when the plasma was tested in the presence of 60% acetonitrile, AI, AII, and aldosterone levels were all stable during the 30-day storage. More important, at room temperature, AI and AII levels in the plasma with no acetonitrile were not stable at all, increasing starting from the first day, and aldosterone was decreased at days 10 and 30. However, in the presence of 60% acetonitrile in the plasma, all 3 analytes showed no significant change over the 30 days, revealing very stable storage at room temperature (**Figure 2**).

Discussion

In this study, we exploited a stable plasma storage method using acetonitrile added to specimens for the analyses of AI, AII, and aldosterone, which were unchanged for up to 30 days at room temperature. This method can spare the reliance on keeping plasma specimens under cold conditions during long-term transportation.

One consideration regarding this approach is that measurements of AI, AII, and aldosterone extracted from this storage method require an independent calibration curve. Otherwise, the results are underestimated by the calibration curve derived from the reference analytes in the original plasma without the addition of acetonitrile, possibly because these competitive immunoassays are subject to specimen matrix interference.[11]

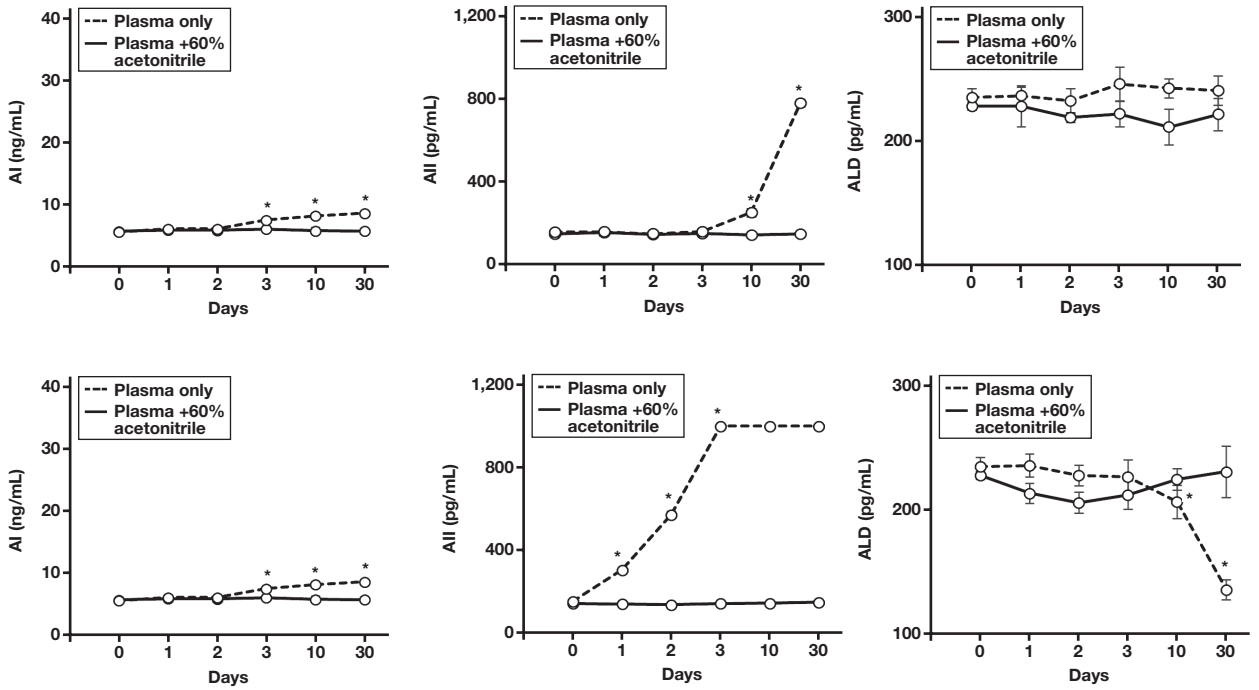


Figure 2

Stable storage of angiotensins and aldosterone in the plasma sample added with acetonitrile. Changes of AI, AII and aldosterone in the plasma containing 60% or no acetonitrile and kept at 4°C or RT (room temperature) over 30 days. Three different plasma samples (AI, AII and ALD) were pooled from collected clinical samples. Half of them were added with 60% acetonitrile and aliquoted, then stored at 4°C or RT. The other half was used as control without addition of acetonitrile. For each time point, three aliquots were used. Samples were harvested at day 0 (immediately), 1, 2, 3, 10 and 30 respectively. All samples were analyzed altogether by an automated chemiluminescence analyzer (Snibe, MAGLUMI 800). Error bars are the standard deviations. Two-way ANOVA were first performed to determine the overall difference between the "plasma only" and "Plasma + 60% acetonitrile" groups (n=3). If a significant difference was found (P-value <0.05), then the one-way ANOVA on each of the two groups was performed. The group with significant difference ($p<0.05$) further received Tukey post-hoc test to find the specific statistically significant data points which were marked with "*".

One limitation of this study is that we only tried 1 level of each analyte for the storage stability experiment. We initially designed 3 concentrations (low, moderate, and high), 3 temperature conditions (4°C, room temperature, and 37°C), and triplicate for each data point (in fact, 4 specimens were planned in case of accidental manual mistakes during experimental procedures). These conditions would require a very large amount of plasma (at least 40 mL). In addition, it is difficult to find specimens with low levels of these analytes simultaneously, and all 3 analytes needed to be measured separately, which would impose a tremendous challenge for the experiments. We therefore finally chose general clinical plasma specimens with a common level of each analyte and 2 regular temperature conditions to simplify the experimental procedure. Lower levels of the analytes are shown in **Figure 1B**, which may indicate that similar results could be generated in the 30-day storage experiment. Nevertheless, lower levels of analytes and higher temperature conditions might be tried in the future to further validate this storage method.

The major limitation of the method used for this study is that although this method provides a simple solution for stable specimen storage, it requires a vacuum concentration step for specimen processing, which may be laborious and generate variations. However, the variation could be controlled because the CVs of the intraday and interday results were small when the experiments were carefully carried out. In addition, acetonitrile precipitates

DOI: 10.1093/labmed/lmaa079

some interference in the sample, clearing it for more accurate measurements of AI, AII, and aldosterone.[12,13] Besides, if the samples are to be measured by liquid chromatography-mass spectrometry/mass spectrometry for the determination of AI, AII, and aldosterone with ^{13}C-labeled analytes as the internal standards[14,15] addition of acetonitrile in the sample does not cause any interference to this type of analysis.

Conclusion

The storage method introduced in this study may be considered when cold conditions are not easily applicable during long-term specimen transportation, enabling centralized studies and wide collaborative research on specimens from remote regions for the renin-angiotensin-aldosterone system analysis. **LM**

Acknowledgments

This study was approved by the ethics committee of the Drum Tower Hospital, which is affiliated with Nanjing University Medical School, China, and was partially supported by the Social Development Program (BE2018601, to BB) from the Science and Technology Department of Jiangsu Province; the General Program of the Health and Family Planning Commission of Jiangsu Province (H2018075, to BB); and the Nanjing Drum Tower Hospital Research Initiation (RE445, to BB). The authors declare no conflicts of interest.

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Clinical Significance of Anti-Modified Citrullinated Vimentin Antibodies in Palindromic Rheumatism

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Laboratory Medicine 2021;52:357-363

DOI: 10.1093/labmed/lmaa095

ABSTRACT

Objective: This study evaluated anti-modified citrullinated vimentin (anti-MCV) performance in determining the clinical picture and outcomes of palindromic rheumatism (PR).

Methods: In a retrospective study, patients with PR with at least 1 year of follow-up diagnosed according to clinical criteria were enrolled. Anti-MCV antibodies were measured, and levels >20 IU/mL were considered positive. Disease prognosis was assessed according to patients acquiring remission and preventing PR from developing into rheumatoid arthritis (RA) or other diseases.

Results: Seventy-six patients with PR with a mean follow-up of 30.57 months (median = 21 months; minimum = 12 months; maximum = 48 months) were included in the study. Anti-MCV antibodies were positive in 69.7% of patients. Metacarpophalangeal (MCP) joint involvement and positive anti-cyclic citrullinated peptides were significantly higher in patients who were anti-MCV-positive, whereas ankle joint involvement was significantly lower. No significant correlation was observed between the anti-MCV titer and the severity of attacks. Remission in patients who were anti-MCV-positive and negative was 75.5% and 78.3%, respectively, with no significant difference. Evolution to RA was observed in only 3.8% of patients who were anti-MCV-positive. No patients who were anti-MCV-negative developed RA.

Conclusion: Except for MCP and ankle joint involvement, anti-MCV was not helpful in determining the clinical picture and outcome of PR.

Keywords: anti-MCV antibodies, palindromic rheumatism, rheumatoid arthritis, prognosis, response to treatment, remission

Palindromic rheumatism (PR) has been depicted as an idiopathic clinical syndrome characterized by intermittent acute attacks of arthritis or peri-arthritis that last a few days and terminate without permanent damage to the joint. Attacks usually start without any precipitating factor, and the most commonly involved joints are the knee, metacarpophalangeal (MCP), wrist, and proximal interphalangeal (PIP) joints.[1] Attacks of PR last from a few hours to a few days and rarely last more than 1 week.[1] There are no clinical, biochemical, or radiographic abnormalities between attacks.[2] Research has shown that PR is not a rare disease. Its prevalence is 0.05–0.6 for every patient with rheumatoid arthritis (RA).[2-4] In addition to impairing the quality of life, PR can evolve to other rheumatic diseases, especially RA.[2] The reported risk of developing RA is 8.7% to 64%.[3,5,6] Therefore, predicting the course of PR is of particular importance. Research has introduced parameters that may predict the progression of PR to RA, including arthritis of the PIP joints, the presence of rheumatoid factor, anti-citrullinated protein antibodies (ACPAs), and genetic markers.[7-9] However, the results are not conclusive.

Research has shown that ACPAs are autoantibodies that are made to counter citrullinated peptides.[10] Anti-cyclic

Abbreviations:

anti-MCV, anti-modified citrullinated vimentin; PR, palindromic rheumatism; RA, rheumatoid arthritis; MCP, metacarpophalangeal; PIP, proximal interphalangeal; ACPAs, anti-citrullinated protein antibodies; anti-CCP, anti-cyclic citrullinated peptide; UA, undifferentiated arthritis; CTDRC, Connective Tissue Diseases Research Center; CTD, connective tissue disease; SLE, systemic lupus erythematosus; SD, standard deviation; DMARDs, disease-modifying antirheumatic drugs.

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citrullinated peptide (anti-CCP) is one of the ACPAs present in 60% to 70% of patients with RA and has a specificity of 90% to 97% for RA.[10] Anti-CCP was included in the 2010 ACR/EULAR classification criteria for the diagnosis of RA.[10] Anti-CCP is also an important predictor of RA development in patients with undifferentiated arthritis (UA).[4,5] Anti-CCP is one of the potent predictors of the outcome in RA.[11] Anti-modified citrullinated vimentin (anti-MCV) antibody is a newer ACPA that detects modified citrullinated vimentin.[12] Vimentin, an intermediate filament expressed in macrophages and mesenchymal cells, is usually not citrullinated, but deamination of this protein occurs in macrophages undergoing apoptosis.[13] Citrullinated vimentin may appear as a result of inadequate clearance of apoptotic material.[13] Anti-MCV has a sensitivity and specificity comparable to anti-CCP for the diagnosis of RA and predicting the progression of UA to RA.[10,14,15]

In 2003, ACPAs were reported in the serum of patients with PR for the first time.[16] Several studies were subsequently performed about the frequency, clinical significance, and prognostic value of anti-CCP in PR.[8,9,16-19] Recently, Cabrera-Villalba et al[20] reported anti-MCV antibodies in patients with PR. In addition, they compared the specificities and isotypes of ACPAs in PR and RA and found fewer specificities and isotypes of these antibodies in RA compared with PR.[20] They reported that IgG against p55 vimentin was present in 59% of patients with RA and in 24% of patients with PR.[20] However, IgM and IgA against this antigen in PR and RA were comparable.[20] No studies have been conducted on the prognostic value of anti-MCV in PR. The purpose of this study was to evaluate the performance of anti-MCV in determining the clinical picture, rate of RA development, and response to treatment in patients with PR.

Materials and Methods

Study Population

In this retrospective study, 76 patients with a diagnosis of PR with at least 1 year of follow-up and 3 visits in a year who were diagnosed according to Guerne and Weisman[21] criteria were enrolled consecutively from the episodic rheumatic diseases clinic of the Connective Tissue Diseases Research Center (CTDRC). Exclusion criteria were other episodic disease with musculoskeletal manifestations such as Behcet disease, familial mediterranean fever, intermittent hydrarthrosis, crystal-induced arthritis and seronegative spondyloarthritis, erosion on joint radiography, and fulfillment of other connective tissue disease (CTD) criteria such as RA and systemic lupus erythematosus (SLE). The study was approved by the ethics committee (approval number: IR.TBZMED.REC.1397.726) of Tabriz University of Medical Sciences, and written informed consent was obtained from all participants before inclusion in the study.

Clinical and Biochemical Measurements

Demographic and clinical characteristics of patients, duration of symptoms until diagnosis, medications, and results of treatment were obtained from patients' records. Stored sera obtained around the time of presentation to the CTDRC clinic were tested for anti-MCV antibodies (IgG), which were measured using commercially available ELISA kits (Orgentec Diagnostika GmbH, Germany). Serum anti-MCV levels >20 IU/mL were considered positive.

Therapies and Outcomes

According to the protocol of our center for the treatment of PR, hydroxychloroquine (5 mg/kg/d) and low-dose prednisolone (5–10 mg/d) were started in all patients. In patients with resistance to therapy, escalating doses of methotrexate (10–25 mg/week) was added or replaced. In patients who were unresponsive, sulfasalazine (1500–2000 mg/d) was added. In patients whose attacks did not come under control, leflunomide (20 mg/d) was added or replaced the methotrexate. After the attacks were controlled, prednisolone was tapered and discontinued. The prognosis of the disease was assessed according to patients acquiring complete, partial, and sustained remission and preventing the disease from developing to RA or other diseases. We determined complete remission to be complete cessation of attacks for 12 weeks and partial remission as at least a 50% decrease in the frequency of attacks for 12 weeks.[5] Sustained remission was defined as a complete cessation of attacks for 1 year. Failure to achieve complete or partial remission (persistent PR) and conversion to RA or other CTDs were considered a failure of treatment.[5]

Statistical Analysis

Statistical analysis was performed using SPSS software version 16.0 (SPSS Inc, Chicago, IL, USA). Normality of variable distribution was evaluated using the Kolmogorov-Smirnov test. Categorical and normally distributed quantitative variables were displayed as numbers (percentages) and means ± standard deviation (SD), respectively. Nonnormally distributed quantitative variables were presented as median (interquartile range). Between-groups comparisons were made by the χ^2 test and independent sample *t*-test, as appropriate. Pearson correlation analysis was performed to evaluate the correlation between anti-MCV titer, the frequency and duration of attacks, and the number of involved joints in each attack. The Kaplan-Meier method was used to compare the outcome of PR in the presence or absence of anti-MCV. A p value $< .05$ was considered statistically significant.

Results

Seventy-six patients with PR were included in this study. The mean ± SD age at the time of diagnosis and the mean follow-up duration were 40.81 ± 12.4 years and 30.57 months (median = 21 months; minimum = 12 months; maximum = 48 months), respectively. Fifty-one patients (67.1%) were women. Anti-MCV antibodies were positive in 53 patients (69.7%). In 4 (17.4%) of 23 patients with a negative anti-MCV test, anti-CCP was positive. The demographic,

Table 1. Demographic, Clinical, and Laboratory Characteristics of Patients at the Time of Anti-MCV Measurement

	Anti-MCV+ (n = 53)	Anti-MCV– (n = 23)	*P* Value
Women (%)	34 (64.2)	17 (73.9)	.289
Age (y), mean ± SD	42.3 ± 11.4	37.4 ± 12.1	.110
Disease duration (mo), mean	24.3 (median = 12; minimum = 6.5; maximum = 180)	28.5 (median = 12; minimum = 6; maximum = 156)	.927
Follow up duration (mo), mean	32.4 (median = 20; minimum = 12; maximum = 56)	36.7 (median = 24; minimum = 12; maximum = 70)	.756
BMI	27.7 ± 5.1	24.9 ± 3.1	.037
Family history of PR or inflammatory CTDs (%)	9 (18)	2 (10)	.378
Frequency of attacks (wks), mean	2.6 (median = 2.0; minimum = 0.2; maximum = 10)	4.3 (median = 2.5; minimum = 0.2; maximum = 12)	.445
Duration of attacks (d), mean	2.5 (median = 2.0; minimum = 0.2; maximum = 13)	2.9 (median = 2.0; minimum = 0.2; maximum = 13)	.847
Number of involved joints in each attack, mean ± SD	1.1 ± 0.3	1.2 ± 0.3	.878
Involved structures			
Knees (%)	36 (67.9)	16 (69.7)	.601
MCP joints (%)	37 (69.8)	10 (43.5)	.022
Shoulders (%)	29 (54.7)	9 (39.1)	.140
Wrists (%)	27 (50.9)	12 (52.2)	.460
Hand PIP joints (%)	28 (52.8)	12 (52.2)	.523
Ankles (%)	14 (26.4)	12 (52.2)	.033
Elbows (%)	12 (22.6)	8 (34.8)	.218
MTP joints (%)	7 (13.2)	3 (13)	.637
Hips (%)	4 (7.5)	3 (13)	…
Foot PIP joints	2 (3.8)	2 (8.7)	…
Peri-articular (%)	11 (20.8)	6 (26.1)	.424
RF (%)	25 (47.2)	7 (30.4)	.107
Anti-CCP (%)	45 (84.9)	4 (17.4)	.001

Anti-CCP, anti-cyclic citrullinated peptide; anti-MCV, anti-modified citrullinated vimentin; BMI, body mass index; CTDs, connective tissue diseases; MCP, metacarpophalangeal; MTP, metatarsophalangeal; PIP, proximal interphalangeal; PR, palindromic rheumatism; RF, rheumatoid factor; SD, standard deviation.

Lab Medicine 2021;52;357–363
DOI: 10.1093/labmed/lmaa095

clinical, and laboratory characteristics of patients with regard to anti-MCV status are shown in **Table 1**. Weight, frequency of MCP joint involvement, and positive anti-CCP in patients who were anti-MCV-positive were significantly higher than in patients who were anti-MCV-nagative (**Table 1**). Ankle joint involvement in patients who were anti-MCV-positive was significantly lower than in patients who were anti-MCV-negative (**Table 1**). No significant differences were observed in other demographic and clinical characteristics of patients who were anti-MCV-positive or -negative (**Table 1**). In comparing the demographic and laboratory characteristics of patients by ACPA status (**Table 2**), we found that in 19 patients (25%) anti-MCV and anti-CCP levels were negative. Except for more involvement of MCP joints and less involvement of ankle, no significant differences were observed in patients with negative or positive ACPAs.

We compared the anti-MCV levels in patients with and without different joint involvement. Anti-MCV titer in patients with MCP joint involvement was significantly higher than in patients without MCP joint involvement (**Table 3**). No significant correlation was observed between anti-MCV titer and the severity of PR attacks (**Table 4**).

We assessed the outcome of treatment in patients with positive and negative anti-MCV. Remission rates (complete and partial) in patients who were anti-MCV-positive and -negative were 75.5% and 78.3%, respectively. No significant differences were observed in the rate of conversion to RA, steroid discontinuation and medication-free remission, and the number of disease-modifying antirheumatic drugs (DMARDs) between the 2 groups during the follow-up period (**Table 5**, **Figure 1**).

Discussion

In the present study, we assessed serum anti-MCV levels in patients with PR to identify whether anti-MCV could be involved in the clinical picture, prognosis, and response to treatment in this disease. Anti-MCV was positive in 69.7% of patients and was positive in 17.4% of patients who were anti-CCP-negative. There were no significant associations between the demographic and clinical manifestations of PR and anti-MCV status except for more MCP and less ankle

Table 2. Comparing Demographic and Clinical Characteristics of Patients by ACPA Status

	Anti-MCV+/Anti-CCP+ (n = 45)	Anti-MCV+ or Anti-CCP+ (n = 12)	Anti-MCV–/ Anti-CCP– (n = 19)	*P* Value
Female/male	26 (57.8)	11 (91.7)	14 (73.6)	.066
Age (y), mean ± SD	42.9 ± 10.4	41.1 ± 16.9	35.61 ± 12.8	.110
Disease duration (mo), mean	24.3 (median = 12; minimum = 6.5; maximum = 180)	28.5 (median = 12; minimum = 6; maximum = 156)	30.1 (median = 12; minimum = 6; maximum = 34)	.927
BMI	28.1 ± 5.2	25.2 ± 3.6	24.9 ± 3.6	.037
Frequency of attacks (wks), mean	2.6 (median = 2.0; minimum = 0.2; maximum = 10)	4.3 (median = 2.5; minimum = 0.2; maximum = 12)	4.5 (median = 2.5; minimum = 1; maximum = 10)	.445
Duration of attacks (d), mean	2.5 (median = 2.0; minimum = 0.2; maximum = 13)	2.9 (median = 2.0; minimum = 0.2; maximum = 13)	3.2 (median = 2.0; minimum = 1; maximum = 5)	.847
Number of involved joints in each attack, mean ± SD	1.1 ± 0.3	1.2 ± 0.3	1.2 ± 0.6	.878
Involved structures				
Knees (%)	30 (66.7)	9 (75)	13 (68.4)	.617
MCP joints (%)	34 75.6	4 (33.3)	9 (47.4)	.015
Shoulders (%)	28 (62.2)	4 33.3	6 (31.6)	.048
Wrists (%)	27 (60)	6 50	11 (57.9)	.944
Hand PIP joints (%)	25 (55.6)	7 (58.3)	12 (63.2)	.788
Ankles (%)	10 (22.2)	7 (58.3)	9 (47.4)	.014
Elbows (%)	11 (24.4)	3 (25)	6 (31.6)	.836
MTP joints (%)	7 (15.6)	0	3 (15.8)	…
Hips (%)	2 (4.4)	3 (25)	2 (10.5)	…
Foot PIP joints	2 (4.4)	0	2 (10.5)	…
Peri-articular (%)	11 (24.4)	0	6 (31.6)	.125

ACPA, anti-citrullinated protein antibody; anti-CCP, anti-cyclic citrullinated peptide; anti-MCV, anti-modified citrullinated vimentin; BMI, body mass index; MCP, metacarpophalangeal; MTP, metatarsophalangeal; PIP, proximal interphalangeal; SD, standard deviation.

Table 3. Comparing Anti-MCV Titer in Patients With and Without Different Joint Involvement

Involved Joints	Anti-MCV Titer	*P* Value
Knee joint involvement		
Yes	332.7 (median = 56; minimum = 2; maximum = 2182)	.744
No	373.3 (median = 45; minimum = 2; maximum = 1000)	
Wrist joint involvement		
Yes	344.5 (median = 40; minimum = 2; maximum = 1075)	.990
No	346.1 (median = 53; minimum = 2; maximum = 2182)	
Shoulder joint involvement		
Yes	447.3 (median = 103; minimum = 2; maximum = 2182)	.095
No	240.1 (median = 40; minimum = 2; maximum = 1075)	
MCP joint involvement		
Yes	461.9 (median = 92; minimum = 2; maximum = 2182)	.008
No	149.3 (median = 31; minimum = 2; maximum = 1198)	
PIP joint involvement		
Yes	408.8 (median = 45; minimum = 2; maximum = 1616)	.419
No	258.8 (median = 56; minimum = 2; maximum = 2182)	
Ankle joint involvement		
Yes	243.6 (median = 26; minimum = 2; maximum = 1198)	.080
No	399.1 (median = 55; minimum = 2; maximum = 2182)	
Elbow joint involvement		
Yes	359.2 (median = 63; minimum = 4; maximum = 1614)	.886
No	340.1 (median = 33; minimum = 2; maximum = 2182)	
Peri-articular involvement		
Yes	438.8 (median = 67; minimum = 4; maximum = 2182)	.732
No	317.7 (median = 54; minimum = 2; maximum = 1616)	

Anti-MCV, anti-modified citrullinated vimentin; MCP, metacarpophalangeal; PIP, proximal interphalangeal.

Table 4. Correlation Between Severity of Attacks and Anti-MCV Titer

	r	*P* Value
Frequency of attacks (wks)	0.017	.889
Duration of attacks (d)	0.103	.378
Number of involved joints in each attack	0.113	.335

Anti-MCV, anti-modified citrullinated vimentin.

joint involvement in patients who were anti-MCV-positive. Furthermore, no significant differences were observed in patients' prognosis and response to treatment between the anti-MCV-positive and -negative groups.

There are limited and conflicting data regarding the prognostic value of ACPAs in PR, and no studies have been performed on the prognostic value of anti-MCV in PR. In a study on 32 patients with PR, Salvador et al[16] found anti-CCP in 56% of patients. No associations were reported between the clinical manifestation of the disease and anti-CCP status.[16] In a previous study on 92 patients with PR, 17% of patients had no response to treatment, and no association was observed between anti-CCP status and the development of persistent disease or RA.[3] In Sanmartí et al,[22] anti-CCP was measured in 71 patients with PR who were followed for a mean period of 7.6 years. Seventy-three percent of patients were were treated using hydroxychloroquine or other DMARDs. The study found that RA, SLE, and other diseases were developed in 22%, 5.6%, and 5.6% of patients, respectively. Progression to RA occurred in 30% of patients who were anti-CCP-positive compared with 15% of patients who were anti-CCP-negative. However, this difference did not reach a statistically significant level, and the sensitivity and specificity of anti-CCP in predicting progression to RA were 69% and 53%, respectively.[22] However, in another study, whereas anti-CCP was positive in 62% of patients with PR paients who progressed to RA, it was positive in only 4.4% of patients who did not progress to RA.[18] Emad et al[19] followed 90 patients with PR for 1 year. The disease evolved to RA in 27% of patients. In univariate regression analysis, hand joint involvement and the presence of anti-CCP were the only predictors. Tamai, Kawakami, Iwamoto, et al[9] prospectively studied 28 patients with PR for a mean period of 38 months. Anti-CCP was positive in 91% of patients who developed RA and only in 18% of patients with persistent PR.[9]

In contrast to most studies about the performance of ACPAs in predicting the progression of PR to RA, our results

DOI: 10.1093/labmed/lmaa095

Table 5. Patients' Prognosis and Response to Treatment (n = 76)

	Anti-MCV+ (n = 53)	Anti-MCV– (n = 23)	*P* Value
Complete remission (%)	37 (69.8)	12 (52.2)	.132
Sustained remission (%)	29 (54.7)	11 (47.8)	.363
Partial remission (%)	3 (5.7)	6 (26.1)	.118
Persistent disease (%)	11 (20.8)	5 (21.7)	.484
Conversion to RA (%)	2 (3.8)	0	…
Time to complete or partial remission (wks)	20.5 (median =12; minimum = 4; maximum = 156)	19.8 (median = 12; minimum = 4; maximum = 94)	.641
Duration of remission	22.5 (median = 15.5; minimum = 4; maximum = 28)	26.5 (median = 16; minimum = 4; maximum =)	.864
Number of DMARDs (%)			
1	34 (64.2)	16 (69.6)	.428
≥2	19 (35.8)	7 (30.4)	
Prednisolone-free remission (%)	15 (28.3)	5 (21.7)	.155
Medication-free remission (%)	6 (11.3)	2 (8.7)	.156

Anti-MCV, anti-modified citrullinated vimentin; DMARDs, disease-modifying antirheumatic drugs; RA, rheumatoid arthritis.

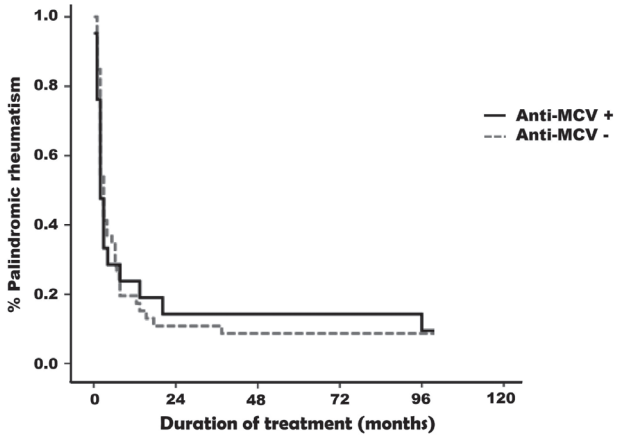

Figure 1

Remission rate in patients with anti-MCV-positive and -negative PR. Anti-MCV, anti-modified citrullinated vimentin; PR, palindromic rheumatism.

did not show a prognostic value for anti-MCV. This finding may have several reasons. For example, selection bias may be the cause. In the majority of patients with PR, progression to RA occurs early in the disease course. Enrollment of a more stable group of patients with long-standing PR, who are less susceptible to RA, may affect the results. According to the Guerne and Weisman criteria,[21] we diagnosed PR in patients who had attacks for at least 6 months before diagnosis. The effect of treatment may be another reason. In our cohort, all the patients were treated with low-dose prednisolone and a combination of DMARDs with step-up strategy to suppress all the attacks. Research has shown that treatment with DMARDs may prevent the progression of RA to PR.[5,22] A significant number of patients with PR did not progress to RA or other CTDs even when ACPAs were positive.[22] In addition, the timing of the ACPA measurement may have had a crucial role. The ACPA response changes with disease evolution,[20] and measuring ACPAs earlier in the disease course may lead to negative results. We measured anti-MCV levels in stored sera obtained at approximately the time of presentation at our center. In most other studies, ACPAs were measured several years after diagnosis.

Conclusion

Except for MCP and ankle joint involvement, anti-MCV may not be helpful in determining the clinical picture and outcome of PR. However, measuring anti-MCV may be helpful in the diagnosis of PR in patients who are anti-CCP-negative. **LM**

Acknowledgments

The results of this article are derived from the specialty degree in internal medicine thesis of coauthor Mahsanam Khaknejad, MD, approved by the Tabriz University of Medical Sciences, Tabriz, Iran. The authors thank all the patients for their participation in this research. This study was financially supported by the Connective Tissue Diseases

Research Center of the Tabriz University of Medical Sciences, Tabriz, Iran.

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Point-of-Care Testing Effectiveness on Blood Donor Hemoglobin Testing

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Laboratory Medicine 2021;52:364-368

DOI: 10.1093/labmed/lmaa102

ABSTRACT

Background: Hemoglobin (Hb) evaluation by point-of-care testing (POCT) identifies borderline or anaemic asymptomatic blood donors. Although quality control checks confirm that this device is fit for use, it is still not clear whether the analyser is performing effectively. A protocol comparing the POCT EKF Diagnostics with the Sysmex XN-550 automated cell counter (ACC) has been designed.

Methods: Various scenarios of Hb measurements from the ACC and the POCT device are compared using the Spearman correlation and Intraclass correlation. The Bland-Altman method was used to analyse the level of agreement between the two devices.

Results: Correlation between the two devices was best observed in the venous vs venous blood scenario.

Conclusion: The POCT device overestimates the Hb levels in capillary blood, meaning that Hb requirements should be adjusted and when feasible testing repeated on venous blood using an ACC. Furthermore, it is suggested thar each Facility determine their own Hb threshold.

For a red blood cell concentrate unit to meet the quality requirements stipulated by the European Directives for Quality of Medicines (EDQM),[1] the hemoglobin (Hb) concentration must be higher than 40 g/dL. This criterion is applicable for male- and female-donated units. At the pre–blood donation phase, the purpose of Hb testing determines the suitability of the final product. This testing also helps identify individuals with abnormally low Hb levels, or those with asymptomatic anemia, particularly those with iron deficiency anemia caused by iron store depletion due to regulation blood donations. Point-of-care-testing (POCT) devices have been used for years for screening Hb in blood banks. These time-saving and easy-to-operate devices may, however, lack reliability in certain circumstances[2] and may require a needle prick of the finger to obtain blood specimens.

Efforts to accurately measure predonation Hb are important for donor safety and component quality. Per the EDQM[1] requirements, abnormal Hb values—namely, greater than 12.5 g/dL and greater than 13.5 g/dL, respectively, for females and males—should be confirmed by performing a full blood count using venous blood. False low-POCT results subject the potential blood donor to another needle prick and prolong the overall blood donation procedure. This circumstance may cause discomfort and inconvenience to the potential donor, who might decide not to donate blood in the future, thus further diminishing the future blood supply. However, a false high-POCT result may lead to unnecessary testing of the donor to evaluate for hematologic conditions such as polycythemia, polycythemia vera, or hemochromatosis and puts the donor at risk of iron-deficiency anemia. Similar to the study conducted by Singh et al[3], this study was designed to ensure that the POCT EKF Hemo Control hemoglobin analyzer (EKG Diagnostic GmbH) is electively detecting donors with low Hb values, with the overall objective of providing other facilities with a protocol to validate the efficiency of their own POCT devices.

Abbreviations:

EDQM, European Directives for Quality of Medicines; Hb, hemoglobin; POCT, point-of-care testing; ACC, automated cell counter; ICC, intraclass correlation; CV, coefficient of variance

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Methods

Ethical approval to perform this study was granted by the Faculty Research Ethics Committee, University of Malta. Venous blood specimens were obtained from healthy adult blood donors during routine voluntary blood donation. A total of 320 prospective donors were included in this study, all of whom were eligible to donate per the EDQM requirements.[1]

Blood specimens were collected from the blood-diversion pouch in a vial containing ethylenediaminetetraacetic acid. These specimens were first screened for multiple possible transfusion-transmitted infections, as part of the daily routine testing stipulated by the EDQM.[1] These specimens were stored at a temperature of 2° to 6°C until tested. Only specimens that had negative screening results were utilized in this study.

A cohort of 31 venous blood specimens known to have a low Hb level (<12.5 g/dL) were provided by the Pathology Laboratory (Hematology) of Mater Dei Hospital, Msida, Malta. These specimens were anonymously provided, meaning that no name, identification number, or results were required.

Before analysis, the blood specimens were brought to room temperature, after which they were gently mixed for 5 minutes using the Multimix Major MM5 Roller (Luckham Ltd). Specimens were visually checked for clots, fibrin strands, or platelet clumps, all of which we subsequently eliminated. Grossly hemolyzed specimens were also eliminated.

Using the automated cell counter (ACC) Sysmex XN-550 (Sysmex Corporation), a complete blood count was performed on each venous specimen, from which the Hb measurement was noted. Subsequently testing was divided into 3 stages, as follows.

Stage 1: Comparing results between the device histories of an ACC and a POCT device. The Hb level in 300 venous donor blood specimens obtained using the ACC was compared with the retrieved corresponding Hb level obtained when testing at the predonation stage on capillary blood using the POCT device.

Stage 2: Comparing results between an ACC and POCT device (same-day testing). The Hb level of another 20 venous blood donor specimens was tested using the same ACC; these specimens were retested on the POCT device on the same day to eliminate any variation caused due to time. POCT readings were performed in triplicate.

Stage 3: Comparing results between an ACC and POCT device when testing specimens with known low Hb. In this stage, we analyzed 31 venous blood specimens known to have a low Hb level, using the ACC and the POCT device. POCT readings were performed in triplicate.

Results

We performed statistical analysis using SPSS software, version 23.0 (IBM Corporation). The data was analyzed for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Correlation between the Hb levels obtained from the ACC and the POCT device was analyzed using the Spearman correlation. The Bland-Altman method and intraclass correlation coefficient (ICC) were used to analyze agreement between the 2 devices. An error bar graph was plotted to assess variation in the POCT device.

On average, the Hb level in capillary blood specimens measured by the POCT device was higher than the Hb level measured in venous blood specimens by the ACC (**Table 1**). This finding was also observed in similar studies conducted by Rudolf-Oliveira et al[4] and Singh et al.[3] In Stage 1, the range for ACC-Hb level was higher than the POCT-Hb level by 1.1 g/dL. However, the mean POCT-Hb exceeded the mean ACC-Hb by 0.19 g/dL. In Stage 2 and Stage 3, the mean Hb value obtained from the POCT device also exceeded the mean ACC-Hb by 0.44 g/dL and 0.24 g/dL, respectively.

The degree of association between the 2 devices was assessed using the Spearman correlation (**Table 2**), which revealed a significantly strong, positive monotonic correlation between the POCT-Hb value in capillary blood and the ACC-Hb value in venous blood ($r = 0.703$; $N = 300$; $P < .001$). The correlation increases on testing venous blood specimens on both devices in stage 2 ($r = 0.947$; $N = 20$; $P < .001$) and stage 3 ($r = 0.911$; $N = 31$; $P < .001$). This finding indicates that the use of venous blood produces more-correlated Hb levels than capillary blood.

Lab Medicine 2021;52;364–368
DOI: 10.1093/labmed/lmaa102

Table 1. Descriptive Statistics for ACC-Hb and POCT-Hb in Stage 1, Stage 2, and Stage 3 Testing

Stage	Variable	Descriptive Statistics						
		No.	Mean (SD)	Median	Minimum	Maximum	Kurtosis	Skewness
Stage 1	ACC-Hb	300	14.61 (1.28)	14.40	11.70	19.6	0.332	0.454
	POCT-Hb	300	14.80 (1.28)	14.80	11.70	18.5	–0.232	0.194
Stage 2	ACC-Hb	20	14.92 (2.15)	14.90	12.60	22.40	7.421	2.297
	POCT-Hb	20	15.36 (2.07)	15.30	13.00	22.47	7.007	2.201
Stage 3	ACC-Hb	31	7.99 (0.98)	8.00	6.50	11.10	2.135	0.944
	POCT-Hb	31	8.23 (1.00)	8.30	6.30	11.67	3.686	1.061

ACC, automated cell counter; POCT, point-of-care testing; Hb, hemoglobin.

Table 2. Spearman Correlation between ACC-Hb and POCT-Hb, for Stage 1, Stage 2, and Stage 3 Testing

Variable			Spearman Correlation	
			POCT-Hb	No.
Stage 1	ACC-Hb	Correlation Coefficient	0.703	300
		Significance	0	
Stage 2		Correlation Coefficient	0.947	20
		Significance	0.000	
Stage 3		Correlation Coefficient	0.911	31
		Significance	0	

ACC, automated cell counter; POCT, point-of-care testing; Hb, hemoglobin.

Table 3. Intraclass Correlation using the 2-Way Mixed-Effect Model between ACC-Hb and POCT-Hb, for Stage 1, Stage 2, and Stage 3

Variable		Intraclass Correlation	
		No.	Average (95% CI)
Stage 1	Correlation	300	0.818 (0.772–0.855)
Stage 2		20	0.986 (0.468–0.997)
Stage 3		31	0.779 (0.547–0.893)

ACC, automated cell counter; POCT, point-of-care testing; Hb, hemoglobin.

Results obtained from the Spearman correlation complemented the results obtained from the ICC (**Table 3**). The ICC revealed that the Hb level in the overall specimens correlated well between the ACC and the POCT device.

The most-correlated values were obtained in stage 2 ($r = 0.986$; $N = 20$), indicating excellent reliability and agreement between the 2 devices when venous blood specimens are subjected to same day testing. Similarly, although to a slightly lesser degree, in stage 1, the Hb levels also correlate well between the 2 devices ($r = 0.818$; $N = 300$), indicating good reliability and agreement between the Hb levels in capillary blood tested on the POCT device and the Hb levels in venous blood tested on the ACC. Singh et al[3] obtained a slightly lower ICC coefficient ($r = 0.770$; capillary POCT-Hb vs venous ACC-Hb) in their study but still had relatively similar results.

Stage 3 yielded the least-correlated Hb levels ($r = 0.779$; $N = 31$); however, the reliability and agreement were still good between the 2 methods when specimens with low Hb levels were tested. These findings imply that the POCT device currently in use at the predonation stage is a reliable instrument, although it may not provide results as accurate as those from an automated analyzer.

The error bar graph observed in **Figure 1** demonstrated variation in POCT-Hb levels at specific ACC-Hb levels. Most of the variation in capillary POCT-Hb levels was obtained at a venous ACC-Hb level between 16.5 g/dL and 17.5 g/dL. There is minimal clinical significance of variation at this range of Hb values. However, a striking variation in the POCT-Hb was observed at an ACC-Hb level of approximately 12.5 g/dL. This finding is clinically significant because an Hb level of 12.5 g/dL is borderline to the eligibility criteria for female donors, and variation at such Hb levels can be critical, possibly leading to blood donation from a donor with anemia or donation-induced anemia.

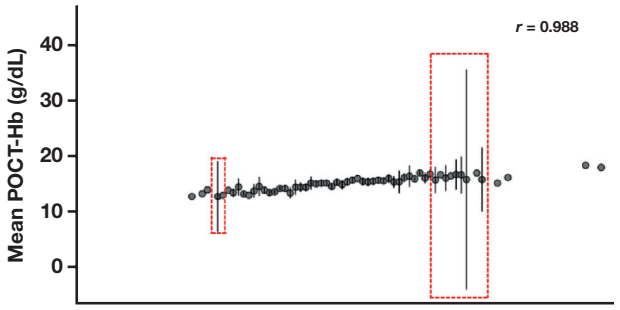

Figure 1

Error-bar graph comparing hemoglobin levels as determined by point-of-care testing (POCT) device vs automated cell counter (ACC).

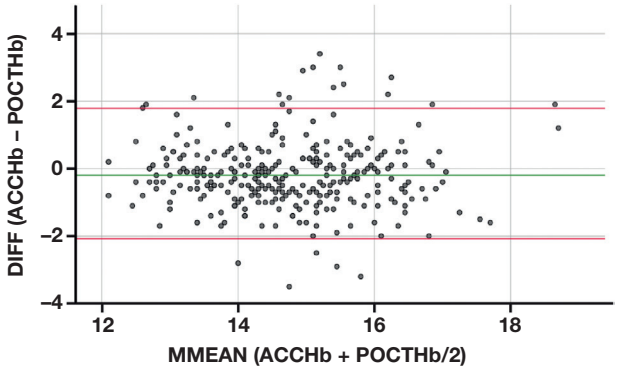

Figure 2

The level of agreement between the 2 tested methods was estimated using a Bland-Altman plot quantifying the bias and range of agreement between the automated cell counter (ACC) and point-of-care testing (POCT) device in testing hemoglobin levels.

Discussion

The accuracy of the POCT device is essential to ensure donor safety. The level of agreement between the 2 tested methods was estimated using a Bland-Altman plot (**Figure 2**), in which the bias and range of agreement ares quantified. The plot shows that most of the data points are randomly distributed around the mean, with a few points falling outside 2 SD. No trends are observed, in the sense that the difference between methods is approximately similar at low and high Hb levels. Nevertheless, the Bland-Altman plot revealed a negative bias of –0.19 g/dL, which means that on average, the Hb level measured in capillary blood by the POCT device was overestimated by 0.19 g/dL, compared with the Hb level measured in venous blood by the ACC. This finding is similar to what Singh et al[3] demonstrated in their study, where the absolute mean difference was found to be 0.22 g/dL. The difference observed by both studies is possibly due to the arterial source of capillary blood resulting in a higher Hb level than in venous blood, as suggested by Daae et al[5] or due to the posture of the donor causing hemoconcentration, as suggested by Schalk et al.[6] Alsos, POCT measurements are highly subjective to the specimen-gathering technique used[2]; these techniques include, but are not limited to, posture (which affects Hb concentration), milking of the finger prior to specimen gathering (aggressive milking will hemolyze the blood), and discarding the first few drops of blood (blood not suitable for testing).

Moreover, the concentration of Hb within the capillary loops fluctuates with skin temperature and depth.[7] The lack of standardization in the POCT specimen-gathering technique is an additional obstacle in obtaining an accurate Hb measurement.

Despite being performed by health care professionals, the finger-prick technique for obtaining capillary blood varies between users. In this study, the limits of agreement between the 2 devices were –2.17 g/dL and +1.78 g/dL, which represent the range within which 95% of differences between measurements by the 2 devices are expected to lie. The clinical implication of these findings is that donors having a borderline Hb level (12.5 g/dL in females and 13.5 g/dL in males) were regarded as eligible when tested using the POCT device but were possibly noneligible when tested using the ACC, due to the apparent overestimation. This situation raises concern because Hb levels decrease by 1 to 2 g/dL after donation (and return to the predonation level after 1 to 2 months) and, in such scenarios, this decrease possibly leads to donation-induced anemia.[8]

Radtke et al[9] reported in their study findings that the lack of agreement between the 2 devices is caused by the specimen type and not because of the method of testing—finger-stick specimens tend to yield slightly higher values than venous specimens. Indeed, these study findings provide strong evidence in favor of this hypothesis because when venous blood was analyzed on the POCT device and ACC, the ICC was higher, compared with the correlation between capillary and venous blood when analyzed on the POCT device and the ACC, respectively.

DOI: 10.1093/labmed/lmaa102

The POCT device was analyzed for imprecision by calculating the variation on repeat testing using a venous specimen. Triplicate readings were obtained in stage 2 and stage 3. In stage 2, the mean coefficient of variance (CV) was of 0.60%, whereas in stage 3, the mean CV was 0.31%. In both stages, the level of imprecision is fairly low; however, the higher CV% observed in stage 2 demonstrates that less-precise Hb levels were obtained in blood specimens from potential healthy donors, compared with in blood specimens from ineligible donors having a Hb level of less than 12.5 g/dL. Nevertheless, the mean CV in stage 2 and stage 3 is within the range specified by the manufacturer, which is less than 2%.

A major limitation in this study was the fact that the data obtained were not clustered based on sex. That finding would have provided a possibility to evaluate more deeply the number of false-positive and false-negative results obtained based on the eligibility criteria for males and females. Also, stage 2 and stage 3 were limited by low sample sizes. Further, the availability of an automated analyzer is not always an option at many blood-drive locations.

Conclusion

Despite providing more-accurate Hb levels than capillary blood, the use of venous blood as part of the Hb screening procedure at the predonation stage is not suitable. Still, critical values should be confirmed using an ACC, although such double testing could pose a problem in the setting of blood drives that are remote to the location of an appropriate ACC. In such cases, the decision to proceed with the blood donation should be at the discretion of the physician on duty. Because in this study the mean difference between the 2 devices was 0.19 g/dL, it is advisable to repeat testing on venous blood using an ACC when the POCT-Hb level in capillary blood is 12.69 g/dL in females and 13.69 g/dL in males—however, doing so will expose the donor to another phlebotomy procedure.

In addition to this recommendation, we advise that each facility perform a similar study, using their own instruments, to define what threshold might be used to trigger testing a venous specimen on an ACC when the POCT value is low. The protocol used in this study may be easily applied to any POCT and ACC device. **LM**

Acknowledgements

We thank the staff at the National Blood Transfusion Service for their cooperation: Special thanks to Prof Alex Gatt, Ms Antionette Mifsud, Mr Neville Borg, and all the staff at the Hematology laboratory, Pathology Department, Mater Dei Hospital, Malta, for providing low-hemoglobin specimens. We also thank Prof Liberato Camilleri, Ms Monique Sciortino, and Dr Stephanie Bezzina Wettinger for their assistance with statistical analysis. This work was supported by the National Blood Transfusion Service, Malta.

Personal and Professional Conflicts of Interest

None reported.

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Incidence of Hepatotoxicity in Iranian Patients With HIV on Antiretroviral Therapies and Its Correlation with Virologic Response to HIV Treatment

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Laboratory Medicine 2021;52:369-374

DOI: 10.1093/labmed/lmaa106

ABSTRACT

Objective: To investigate hepatotoxicity in Iranian patients with HIV to assess the association between virologic response to HIV treatment and serum alanine aminotransferase (ALT).

Methods: This study was conducted with 200 control patients, 75 patients with HIV naïve to antiretroviral therapy (ART), and 443 patients who received ARTs with virologic response (≤1000 copies/mL) or virologic treatment failure (>1000 copies/mL). Serum ALT level and HIV viral load were determined in all patients.

Results: Patient ALT levels were significantly higher than those of control patients (45.1 ± 44.4 IU/L vs 23.8 ± 5.4 IU/L). Compared to patients who were ART-naïve, patients with ART experience had significantly higher ALT levels (38.2 ± 26.2 IU/L vs 46.3 ± 46.7 IU/L), and severe hepatotoxicity was only detected in those with ART experience (8 patients, 1.8%). Mean ALT had no significant difference between virologic response/failure groups. The ALT activity and HIV load had a negative correlation coefficient, but it was not significant.

Conclusion: Periodic monitoring for the possibility of hepatotoxicity is highly recommended in all patients with HIV, especially in those receiving ART treatment.

Keywords: HIV, hepatotoxicity, ALT, viral load, ART, ART-naïve

Emerging viral diseases continue to pose an important public health concern for both the developed and developing world and have caused great catastrophes in human history.[1] Research has shown that HIV is a bloodborne virus typically transmitted via sexual intercourse and attacks immune cells including T-helper lymphocyte, TCD4+, macrophages, and dendritic cells, which are also crucial factors for parasite clearance and act as the main arm of immunity against different tumors.[2–8] It is grouped to the genus *Lentivirus* within the family of Retroviridae and stores its genetic information as RNA that is responsible for causing AIDS, which is the late stage of the spectrum of disease.[9–11] Whereas combination antiretroviral therapies (ARTs) have shown efficacy in improving immune function and survival rate of patients with HIV and reduced the risk for development of AIDS, metabolic disorders such as liver disease are becoming increasingly important sources of morbidity and mortality.[10,12–15]

In addition, the elevation of liver enzymes in the serum of patients with HIV is a common event because of liver damage; therefore, high levels of liver enzymes such as alanine aminotransferase (ALT) and aspartate

Abbreviations:

ALT, alanine aminotransferase; ART, antiretroviral therapy; HBV, hepatitis B virus; HCV, hepatitis C virus.

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aminotransferase in the blood are a prime indicator of liver injuries.[16–19] It has been suggested that drug treatment can be associated with hepatotoxicity and ARTs are no exception, but a number of abnormalities are not related to the use of an ART regimen and may result from coinfection with viral hepatitis.[16,19,20] On the other hand, HIV infection can directly infect hepatocytes and may have an impact on the pathogenesis of liver disease; however, the mechanisms are not fully elucidated.[21,22] Therefore, it is difficult to separate drug-induced hepatotoxicity from the hepatic damage induced by viral infections; however, the evaluation of potential liver injury represents a critical step in the management of patients with HIV.[19,20]

The HIV viral load is considered as the gold standard for management and monitoring of patients with HIV and precisely identifies treatment failure and patients who are nonadherent to therapy regimens. The current World Health Organization guidelines and previous studies have suggested that viral suppression is achieved in patients on ARTs (for more than 6 months) when plasma HIV viral load is below the threshold of virologic response (≤1000 copies/mL).[23–26] In addition, a linear correlation has been observed between aminotransferase levels and HIV viral load.[9,21,22]

To gain a better insight into the effect of HIV and ARTs on the liver function of Iranian patients with HIV, the current study investigated the frequency of hepatotoxicity in 3 groups of Iranian patients with HIV on ARTs—those who were virologic responders, those with virologic treatment failure, and those who were ART-naïve—and assess the association between ARTs and HIV infection with the serum ALT level.

Materials and Methods

A total of 518 Iranian outpatients with HIV were enrolled in this study, which was referred to the special diagnostic clinic affiliated with Shiraz University of Medical Sciences, Shiraz, Iran. The inclusion criteria were being HIV-infected with negative antibodies against hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. Using standard questions, pregnant women, those with known liver diseases or a history of alcohol consumption, and patients using drugs, which may cause fatty liver or liver toxicity, were excluded. The patients' group was divided into 3 subgroups: ART-naïve (75 patients), those with virologic response (289 patients), and virologic treatment failure (154 patients) who had previously received ART for more than 6 months. The control group was defined as 200 healthy blood donors who were confirmed to have no evidence of HIV, HBV, or HCV infections. The research protocol was approved by the ethics committee of Shiraz University of Medical Sciences, Shiraz, Iran.

Specimen Collection

Five mL of venous blood specimens were collected from each individual in nonheparinized tubes and centrifuged at 3000 rpm for 5 minutes to separate the serum. The serums were then transferred into new tubes and stored at –70°C until further use.

Quantification of HIV and Biochemical Tests

Viral RNA was extracted from 200 μL of serum using the Invisorb Spin Virus Mini Kit (Stratec Inc, Berlin, Germany) according to the manufacturer's protocol. High-quality RNA was finally eluted in 100 μL of Diethylpyrocarbonate (DEPC)-treated sterile water. The serum HIV-RNA load was determined by the sensitive and accurate real-time polymerase chain reaction assay using the Altona RealStar HIV RT-PCR Kit (Altona Diagnostics, Germany). The serum level of ALT was measured in all patients by an automated biochemical analyzer with commercially available reagents from Pars Azmoon Inc (Pars Azmoon, Tehran, Iran).

Statistical Analysis

All statistical analyses were performed using the SPSS software version 21 (IBM Corporation, Armonk, NY, United States). Variables were checked for normality by the Kolmogorov-Smirnov test. Categorical variables were given as the number and percentage and compared with a χ^2 test. Continuous variables were expressed as mean ± standard deviation, and the comparison of mean values between the groups was performed by *t*-test. A *P* value of <.05 was considered to be statistically significant.

Results

The patient group was 309 (59.7%) men and 209 (40.3%) women ages 18 to 61 years (mean age, 38.3 ± 13.2 years).

The control group of this study comprised 123 (61.5%) men and 77 (38.5%) women, with a mean age of 37.1 ± 10.8 years (aged 21–63 years). There were no statistically significant differences between the patient and control groups with respect to age and sex (P >.05). The HIV viral load was detected in 289 (65.2%) patients undergoing treatment with a mean level of 2.9×10^5 copies/mL and in all patients who were ART-naïve (mean, 8.1×10^5 copies/mL).

Elevated ALT activity was defined as ALT values >40 IU/L, which were detected in 166 patients (32%), the majority of whom (88%) were classified as patients who had received ART. Mean ALT activity in patients with HIV was 45.1 ± 44.4 IU/L, which was significantly higher than that observed in the control group (23.8 ± 5.4 IU/L; P <.0001). There was a significant difference between the mean ALT activity of patients who were ART-naïve (38.2 ± 26.2 IU/L) and those who had received ART (46.3 ± 46.7 IU/L; P =.031), and both groups had higher ALT activity than the control participants (P <.0001). Furthermore, our results revealed no significant difference in the mean values of ALT between the virologic responder group (42.2 ± 37.1 IU/L) and the virologic treatment failure group (48.5 ± 51.1 IU/L; P =.178), but the differences were highly significant when compared to those of the control group (P <.0001). A statistically significant difference was observed in the ALT activity of patients who were ART-naïve and those in the virologic treatment failure group (P =.016), but not between ART-naïve and patients in the virologic responder group (P =.396).

According to the definition and classification of hepatotoxicity,[27,28] the incidence of hepatotoxicity was common in our series of patients and diagnosed in 125 partients (24.1%); however, the rate of severe hepatotoxicity was low and observed in 8 (1.5%) patients who had received ART (**Table 1**).

In addition, the rate of hepatotoxicity was similar between the virologic response and virologic treatment failure groups (P =.187). Patients' ALT activity and HIV viral load had a negative correlation coefficient (–0.102), but this relationship was not significant (P =.088). Finally, no significant differences were observed in either the serum ALT level or the rate of hepatotoxicity with respect to the patients' sex (P >.05).

Discussion

Our results suggest that HIV infection and ART can be plausible causes of liver injury, at least partially. This study is the first survey conducted about the ALT activity of Iranian patients with HIV who are ART-naïve/ART-experienced without HBV or HCV coinfections. To date, most studies that show ART-induced hepatotoxicity in patients with HIV patients have been conducted in the presence of other related factors such as alcohol abuse, viral hepatitis, or tuberculosis,[29–32] whereas the present study excluded the mentioned factors.

We found that the serum level of ALT as a liver injury marker was elevated in Iranian patients with HIV receiving ART and patients who were ART-naïve compared with healthy control patients. In the absence of other risk factors of hepatotoxicity like HBV and HCV in our series of patients, liver injuries may be induced directly by HIV infection and indirectly by ART therapy. Although the liver cells are not the exact site of HIV replication, some evidence shows that replication of HIV in hepatocytes occurs at relatively low levels.[21,22,33,34] Moreover, the elevation of ALT levels in patients on ART

Table 1. Classification of Hepatotoxicity Among Patients with HIV

Study Groups		Severity of Hepatotoxicity				
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
		<1.25 × ULN (1–50 IU/L)	1.25–2.5 × ULN (50–100 IU/L)	2.6–5 × ULN (104–200 IU/L)	5.1–10 × ULN (204–400 IU/L)	>10 × ULN (>400 IU/L)
ART-naïve, % (n=75)		60 (80)	12 (16)	3 (4)	NA	NA
ART-experienced, % (n=443)	Virologic response (n=289)	213 (73.6)	49 (17)	19 (6.6)	8 (2.8)	NA
	Virologic failure (n=154)	120 (77.9)	23 (14.9)	11 (7.2)	NA	NA
	Total	333 (75.1)	72 (16.3)	30 (6.8)	8 (1.8)	NA

ULN, upper limit of normal.

Lab Medicine 2021;52;369–374
DOI: 10.1093/labmed/lmaa106

therapy compared with patients who were ART-naïve suggests that ARTs can also damage the liver and cause hepatotoxicity. In accordance with our results, the elevation of transaminases in patients with HIV has been reported in several studies.[35–37]

Furthermore, our results revealed that the serum levels of ALT in both ART-experienced groups were higher than those observed in patients who were ART-naïve or healthy control patients, which is in accordance to those reported by Odiba, Onosakponome, et al[35]; Usman et al[36]; Agbecha and Ikyernum[37]; and Adiga and Malawadi.[38] In contrast, Osakunor et al[39] conducted a case-control study on 2 groups of patients with HIV who were receiving ART and who were ART-naïve in Ghana and found that ART had minimal effects on hepatotoxicity and ALT activity. In line with the current results, the elevation of ALT in patients with HIV who were ART-naïve has also been reported by some authors[35–37,40]; however, this finding was not the case in Indian patients with HIV who were ART-naïve.[38] The differences can be attributed to population genetics, different demographic and lifestyle factors, and the number of participants enrolled in the studies.

In this study, elevated ALT level was present in 32% of patients with HIV, including 146 (33%) and 20 (26.7%) patients in the ART-experienced and ART-naïve groups, respectively. The rate of ALT abnormality in the current study was lower than that observed in patients with HIV who had received ART in Nigeria (74.2%)[35] and similar to that in Cameroonian patients (22.6%).[41] On the other hand, the elevation of ALT level in our patients with HIV who were ART-naïve (26.7%) was higher than that observed in patients who were ART-naïve in Tanzania (13%)[42] and North America (15%).[43]

The results of the present study showed that in Iranian patients with HIV, mild hepatotoxicity (grades 1 and 2) was observed in 23.1% and 20% of patients who were receiving ART and those who were ART-naïve, respectively. However, the rate of severe hepatotoxicity (grades 3 and 4) was low and observed in only 8 patients (1.5%) receiving ART, suggesting that despite the benefits of the ART regimen, it has an adverse effect on hepatocytes.

In line with our results and regardless of the type of treatment regimen, some studies have reported a higher rate of mild hepatotoxicity (16.2%–100%) and a low frequency of severe hepatotoxicity (0%–17.7%) in patients with HIV receiving ART.[17,38,44–51] The lower rate of severe hepatotoxicity may result from the absence of other traditional risk factors such as viral hepatitis coinfections, alcohol abuse, and differences in treatment regimens and duration of therapy. According to our results, the periodic monitoring of patients with HIV, especially those receiving ART, is recommended to trace the possibility of hepatotoxicity; however, the discontinuation or alteration of ART in such patients is questionable and requires further studies.

Previous studies conducted on liver enzyme elevation in patients with HIV mono-infection revealed a significant positive correlation between elevations of ALT and increase in HIV viral load, independent of the use of ART.[10,21,22,52] In the current study, the elevation of ALT was not correlated with HIV viral load, which is in contrast with some studies performed on patients with HIV who were treatment-naïve.[21,22] There were no significant differences in either the serum ALT level or the rate of hepatotoxicity in patients with HIV with respect to male sex, which is in agreement with studies reporting similar trends between males and females[45] and in contrast with rates observed in Tanzanian[42] and Cameroonian[53] populations.

Conclusion

Because the elevated level of serum ALT was observed in different groups of patients with HIV who were receiving ARTs or who were ART-naïve, it can be concluded that HIV infection and ART therapy may have an impact either directly or indirectly on hepatocyte function; however, the mechanisms are not fully elucidated. In other words, the elevation of the ALT enzyme in patients receiving ART is an important risk factor associated with hepatotoxicity; therefore, monitoring patients with HIV for the possibility of ALT abnormality and hepatotoxicity is highly recommended. Further studies with long-term follow-up are needed to elucidate the role of HIV infection and ART regimens in the hepatotoxicity of patients with HIV. Although this study excluded patients with known liver diseases as much as possible, the results may be confounded by those who had undiagnosed liver disorders such as nonalcoholic steatohepatitis or by drug-induced ALT elevation in the ART-experienced group; therefore, we suggest assessing these points in future studies. **LM**

Acknowledgments

The authors thank the vice-chancellor of research at Shiraz University of Medical Sciences, Shiraz, Iran, for supporting the research (grant number 1396-01-59-14321). This manuscript is related to part of a master student's thesis. The authors thank the Center for Development of Clinical Research of Nemazee Hospital and Dr. Shokrpour for help in revising the English of the manuscript.

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DOI: 10.1093/labmed/lmaa106

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Evaluation of Serum GDF15, AFP, and PIVKA-II as Diagnostic Markers for HBV-Associated Hepatocellular Carcinoma

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Laboratory Medicine 2021;52:381-389

DOI: 10.1093/labmed/lmaa089

ABSTRACT

Objective: To evaluate the potential diagnostic value of growth differentiation factor 15 (GDF15) alone and its combination with protein induced by vitamin K absence-II (PIVKA-II) and alpha-fetoprotein (AFP) for hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC).

Methods: Serum levels of GDF15, PIVKA-II, and AFP were measured in 110 patients with HBV-associated HCC, 70 patients with HBV-related liver cirrhosis (LC), 70 patients with chronic hepatitis B (CHB), and 110 healthy patients.

Results: Serum GDF15 was positively related to the levels of PIVKA-II and AFP in patients with HCC ($r = 0.352$ and $r = 0.378$; all $P < .0001$). When the receiver operating characteristic (ROC) curve was plotted for patients with HCC vs all control patients, serum GDF15 had diagnostic parameters of an area under the curve (AUC) of 0.693, a sensitivity of 67.30%, and a specificity of 66.70%, which were lower than parameters for PIVKA-II and AFP (all $P < .0001$). When the ROC curve was plotted for patients with HCC vs patients with LC, the combination of GDF15 and PIVKA-II had the highest diagnostic accuracy of AUC and specificity as compared with other combinations (all $P < .0001$).

Conclusion: We found that GDF15 is a potent serum marker for the detection of HBV-associated HCC and that PIVKA-II combined with GDF15 can improve diagnostic accuracy for HBV-associated HCC.

Keywords: growth differentiation factor 15, protein induced by vitamin K absence or antagonist II, alpha-fetoprotein, hepatitis B virus, hepatocellular carcinoma, diagnosis

Infection with hepatitis B virus (HBV) is an extremely important public health problem worldwide. It is estimated that approximately 2 billion people worldwide are infected with HBV and that more than 350 million people are chronic carriers of HBV.[1] Eventually, approximately 15% to 40% of infected patients (mainly those with chronic hepatitis B [CHB]) will develop liver failure, liver cirrhosis (LC), or hepatocellular carcinoma (HCC).[2] Infection with HBV causes 500,000 to 1.2 million deaths each year and is the 10th leading cause of death worldwide.[3] Incidence of HCC has increased worldwide, and the disease has become the fifth most common cancer, causing 30 to 50 million deaths per year.[4] It is the fourth most common cancer and the third leading cause of cancer-related death in China, where approximately 50% of patients with new cases of HCC each year worldwide reside.

Serum alpha-fetoprotein (AFP) and serum protein induced by vitamin K absence-II (PIVKA-II) have been widely used serum biomarkers in the diagnosis and prognosis monitoring of HCC in recent years. Levels of AFP are the most commonly used markers for the clinical diagnosis of HCC,[5] but because of the poor sensitivity and specificity of AFP, its diagnostic efficiency is limited and it is no longer recommended by the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases.[6,7] After AFP, PIVKA-II is the most widely used marker for the

Abbreviations:

GDF15, growth differentiation factor 15; PIVKA-II, protein induced by vitamin K absence-II; AFP, alpha-fetoprotein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LC, liver cirrhosis; CHB, chronic hepatitis B; ROC, receiver operating characteristic; AUC, area under the curve; HCV, hepatitis C virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase; ALB, albumin; TBIL, total bilirubin; DBIL, direct bilirubin; FIB, fibrinogen; ELISA, enzyme-linked immunosorbent assay; CI, confidence interval.

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diagnosis of HCC, and a great deal of the case-controlled studies comparing AFP with PIVKA-II for the diagnosis of HCC have shown an increased sensitivity and specificity of PIVKA-II over AFP and indicated that the combination of the 2 could improve the disease detection rate.[8-11]

On the other hand, a recent report speculated that the growth differentiation factor 15 (GDF15) level has a prognostic value in patients with HCC. The serum GDF15 level can be used to evaluate the status of liver fibrosis and guide clinical medication.[12] Although previous research on GDF15 has focused on inflammatory disease, there is increasing evidence to indicate that GDF15 may be a key cytokine linking inflammation and cancer, not least because a malignant cycle of inflammation and repair promotes tumorigenesis.

No correlation has been reported between these 3 biomarkers and the possible relationships between them. Therefore, the purpose of this study was to explore the possible relationship between GDF15, AFP, and PIVKA-II in patients with HCC and to evaluate the clinical utility of GDF15 as a reliable marker for the early detection of HCC and the benefits of combining GDF15 with PIVKA-II and AFP for the detection of HCC.

Materials and Methods

Patients and Healthy Control Patients

Only male patients were included in this study—on the one hand to reduce variables and improve the accuracy of the results, and on the other hand because the number of men in China suffering from liver disease caused by HBV is much higher than that of women, especially HCC caused by HBV. All patients were recruited from January 2019 to November 2019 in the Department of Oncology and the physical examination center at Renmin Hospital of Wuhan University (Wuhan, China) (http://www.rmhospital.com/). A total of 360 patients were divided into a HBV-infection-related HCC group (110 patients), a HBV-related LC group (70 patients), a CHB group (70 patients), and the healthy control group (110 patients). Patients with HCC were diagnosed based on the guidelines of the Chinese Society of Hepatology and the Chinese Society of Infectious Diseases, Chinese Medical Association. All the patients with HCC were confirmed with liver biopsy or X-ray computed tomography or magnetic resonance imaging. All patients with HCC, LC, or CHB with other serious illnesses, such as other infectious diseases, organ tumors, lung diseases, severe kidney disease, or severe cardiovascular and cerebrovascular disease, were excluded. All healthy control patients tested negative for HBV, hepatitis C virus (HCV), syphilis, and HIV, and all biochemical tests were normal.

This study was reviewed and approved by the Medical Ethics Review Committee of Renmin Hospital, Wuhan University. All patients approved and signed a written informed consent in accordance with the policies of the Renmin Hospital of Wuhan University Ethics Committee.

Specimen Collection

Plasma specimens were obtained in the morning after fasting for at least 8 hours. Venous blood specimens were collected into a tube with a separation of glue and centrifuged at 3500 rpm/min for 15 minutes at room temperature. Serum specimens were collected and stored at –70°C until analysis.

Laboratory Analyses

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), albumin (ALB), total bilirubin (TBIL), and direct bilirubin (DBIL) were measured using an automatic biochemistry analyzer, the ADVIA 2400, through enzymatic methods (Siemens, Germany). Fibrinogen (FIB) and D-dimer were tested using the SysmexCA-7000 (Kobe, Japan). We tested HBV DNA using the ABI ViiA7 real-time fluorescent quantitative polymerase chain reaction system. The AFP was detected using the Siemens ADVIA Centaur CP (Siemens, Germany), and PIVKA-II was determined using the LUMIPULSE G1200 (Fujirebio Inc, Japan).

To test human GDF15 levels in serum, we used commercial enzyme-linked immunosorbent assay (ELISA) kits purchased from R&D (Quantikine, catalog numbers DY957, DY008, R&D Systems). The range of values detected by this assay was 7.8 pg/mL to 500 pg/mL, and the specimens were diluted 10 times for testing. All measurements of plasma GDF15 were repeated twice for each specimen. The principle of the kit is based on indirect sandwich ELISA technology, which uses captured antibodies and biotinylated detection antibodies to achieve capture and detection purposes, respectively.

Statistical Analysis

Statistical analysis was performed with SPSS version 20.0 (IBM Corp, NY) and MedCal 15.2.2 (Ostend, Belgium), and figures were made using GraphPad Prism 6.0 (GraphPad Software, Inc, La Jolla, CA). The Kolmogorov-Smirnov test was used to assess normality. Normally distributed continuous variables were presented as mean ± standard deviation. Skewed data were expressed as median (interquartile range). Significant differences of clinicopathological parameters among the groups were determined using an analysis of variance, Kruskal-Wallis test, or χ^2 test based on the type of data. A 2-tailed least significant difference (LSD) was conducted to compare the differences between the groups for normally distributed data. Pearson correlation was used to analyze the correlation of indicators. Receiver operating characteristic (ROC) curve analysis was used to analyze the diagnostic value of GDF15, PIVKA-II, and AFP in patients with HCC. Finally, 2-sided *P* values <.05 were considered statistically significant.

Results

Characteristics of the Study Population

The characteristics of all patients are shown in **Table 1**. In the study, all study participants were of similar age (*P* >.05). According to the analysis of the hepatic biochemical index, ALT, AST, ALP, GGT, ALB, TBIL, DBIL, FIB, and D-dimer levels were significantly different among the healthy control patients and CHB, LC, and HCC groups (all *P* <.001). The HBV DNA levels in patients with CHB were significantly higher than those in the LC and HCC groups (*P* <.001).

Serum Concentrations of GDF15, AFP, and PIVKA-II in Different Groups

To further assess the serum levels of GDF15, AFP, and PIVKA-II in healthy control patients and patients with CHB, LC, and HCC, we performed the Kruskal-Wallis test. As described in **Table 2**, GDF15 levels in patients with CHB ([693.97 (371.03–1408.87) pg/mL], LC[1696.71 (734.93–2879.77) pg/mL], and HCC [1218.22 (641.32–2453.64) pg/mL] were higher than in healthy control patients [297.35 (156.52–613.23) pg/mL]; *P* <.05, *P* <.001, and *P* <.001, respectively); compared with patients with CHB, the levels of GDF15 in patients with LC and patients with HCC were significantly increased (*P* <.05 and *P* <.001, respectively), but there was no significant difference in GDF15 levels between patients with LC and patients with HCC (*P* >.05). Serum AFP levels in patients with CHB [4.40 (2.25–22.60) ng/mL], LC [8.60 (2.70ng/mL–63.20) ng/mL], and HCC [75.70 (7.70–1334.95) ng/mL] were higher than those in healthy control patients [3.30 (2.23–4.78) ng/mL]; *P* <.05, *P* <.001, and *P* <.001, respectively), and AFP levels in the CHB and LC groups were observably lower than those in the HCC group (all *P* <.001). Finally, serum PIVKA-II levels in the HCC group were significantly higher than in the other 3 groups (all *P* <.001).

Serum Concentrations of GDF15, AFP, and PIVKA-II at Different HCC Stages and Liver Function Grades

To evaluate the changes in GDF15, AFP, and PIVKA-II levels in the progression of HCC, patients with HCC were divided

Table 1. Basic Characteristics of the Study Population

Characteristics	Healthy Control Patients (n = 110)	CHB (n = 70)	LC (n = 70)	HCC (n = 110)	*P* Value
Age (y)	54.63 ± 1.30	54.37 ± 1.53	53.88 ± 1.29	55.62 ± 1.93	>.05
ALT (U/L)	27 (15–31)	135 (24–365)	42 (26–76)	33 (25–49)	<.001
AST (U/L)	20 (15–21)	93 (25–217)	47 (31–89)	43 (30–69)	<.001
ALP (U/L)	68.70 (63.50–21.00)	97.80 (71.95–143.50)	98.60 (75.00–144.50)	119.50 (86.43–172.70)	<.001
GGT (U/L)	26 (16–53)	73 (34–156)	69 (29–105)	81 (43–180)	<.001
ALB (g/L)	43.90 (43.27–44.60)	38.90 (31.80–42.10)	32.80 (27.80–37.20)	38.10 (34.20–41.60)	<.001
TBIL (μmol/L)	9.80 (9.10–16.10)	23.10 (15.95–74.05)	26.70 (15.70–51.80)	19.60 (13.53–31.08)	<.001
DBIL (μmol/L)	3.05 (2.51–4.90)	8.20 (5.40–60.19)	11.60 (6.60–33.15)	7.05 (4.50–10.50)	<.001
FIB (g/L)	2.64 (2.41–3.08)	2.00 (1.74–2.73)	1.98 (1.46–2.27)	2.79 (2.10–3.76)	<.001
D-dimer (mg/L)	0.20 (0.11–0.33)	0.33 (0.17–0.84)	0.73 (0.27–4.18)	0.89 (0.40–2.48)	<.001
HBV DNA (IU/mL)	NA	33,800 (342–673,500)	1350 (58–49,500)	87 (43–5875)	<.001

ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B; DBIL, direct bilirubin; FIB, fibrinogen; GGT, gamma-glutamyl transpeptidase; HCC, hepatocellular carcinoma; LC, liver cirrhosis; NA, not applicable; TBIL, total bilirubin.
Data are presented as means (SD), median (interquartile range), or percentage.

DOI: 10.1093/labmed/lmaa089

Table 2. Comparison of Serum Levels of GDF15, AFP, and PIVKA-II in Healthy Control Patients and Patients with CHB, LC, and HCC

Group	Number	GDF15 (pg/mL)	AFP (ng/mL)	PIVKA-II (mAU/mL)
Healthy control patients	110	297.35 (156.52–613.23)	3.30 (2.23–4.78)	28.50 (24.00–34.00)
CHB	70	693.97 (371.03–1408.87)[a]	4.40 (2.25–22.60)[a]	23.00 (18.00–34.00)
LC	70	1696.71 (734.93–2879.77)[b,c]	8.60 (2.70–63.20)[b]	27.00 (21.00–44.00)
HCC	110	1218.22 (641.32–2453.64)[b,d]	75.70 (7.70–1334.95)[b,c,e]	592.00 (97.00–5493.00)[b,c,e]

AFP, alpha-fetoprotein; CHB, chronic hepatitis B; GDF15, growth differentiation factor 15; HCC, hepatocellular carcinoma; LC, liver cirrhosis; PIVKA-II, protein induced by vitamin K absence -II.
Data are presented as median (interquartile range).
[a,b]Compared with the healthy control patients, P <.05, P <.001.
[c,d]Compared with the CHB group, P <.001, P <.05.
[e]Compared with the LC group, P <.001.

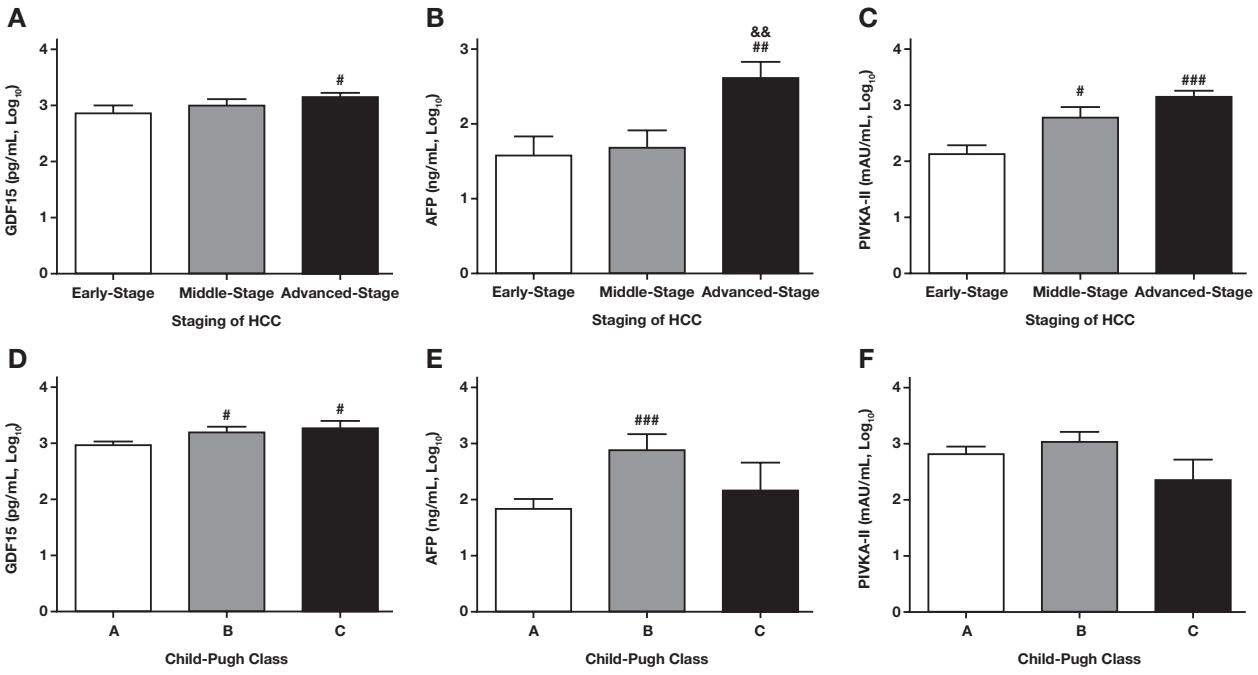

Figure 1

Serum levels of GDF15, AFP, and PIVKA-II according to HCC stage and Child-Pugh class in patients with HCC. Image A-C: GDF15, AFP, and PIVKA-II levels in different HCC stages. Image D-F: GDF15, AFP, and PIVKA-II levels in different Child-Pugh classes. HCC, hepatocellular carcinoma; GDF15, growth differentiation factor 15; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence-II. All data presented in log10 transformed data. [#, ##, ###]Compared with patients with early-stage HCC, P <.05, P <.01, P <.001; [&&]compared with patients with middle-stage HCC, P <.01; [#, ###]compared with patients in class A, P <.05, P <.001.

into early-stage (32 patients), middle-stage (39 patients), and advanced-stage (39 patients) HCC, according to the Barcelona Clinic for Liver Cancer. We found that GDF15 levels in patients with advanced-stage HCC (3.16 ± 0.06 pg/ml) were higher than those in patients with early-stage HCC (2.89 ± 0.10 pg/mL; P <.05; **Figure 1**, Image A). Compared with early- and middle-stage HCC, levels of AFP were significantly higher in patients with advanced-stage HCC (2.62 ± 0.20 pg/mL vs 1.58 ± 0.24 pg/mL and 1.68 ± 0.23 pg/mL, respectively; P <.01; **Figure 1**, Image B). The PIVKA-II levels were increased in patients with middle-stage and advanced-stage HCC, compared with those in patients with early-stage HCC (2.78 ± 0.12 mAU/mL and 3.15 ± 0.12 mAU/mL, respectively, vs 2.15 ± 0.14 mAU/mL; P <.05 and P <.001; **Figure 1**, Image C). According to Child-Pugh class in patients with HCC, class B and class C patients

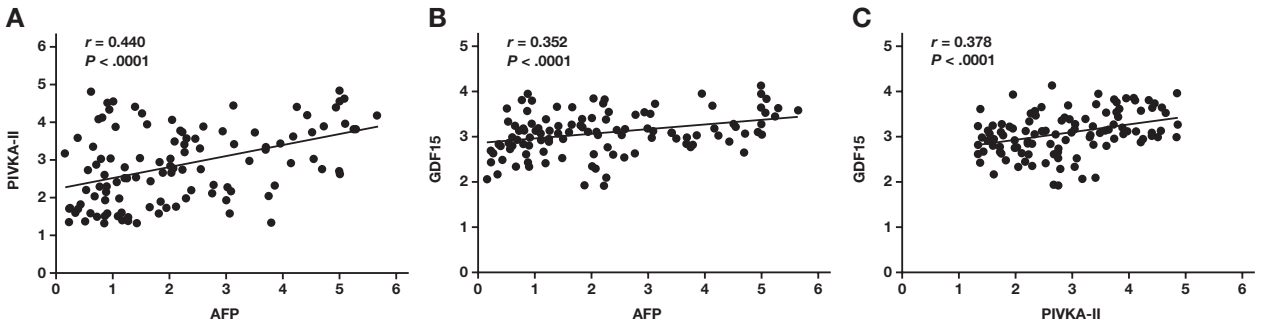

Figure 2

Scatter diagrams illustrating the correlations between PIVKA-II, AFP, and GDF15 (all based on $\log_{10}$ transformed data). **A,** Correlations between PIVKA-II and AFP. **B,** Correlations between GDF15 and AFP. **C,** Correlations between GDF15 and PIVKA-II.

had higher GDF15 levels than class A patients (3.23 ± 0.08 mAU/mL and 3.29 ± 0.11 mAU/mL, respectively, vs 2.99 ± 0.05 mAU/mL; all P <.05; **Figure 1**, Image D). In addition, AFP levels were increased in class B patients compared with class A patients (2.91 ± 0.27 ng/mL vs 1.86 ± 0.16 ng/mL; P <.01; **Figure 1**, Image E).

Correlations Among PIVKA-II, AFP, and GDF15

To further evaluate the correlations among PIVKA-II, AFP, and GDF15, the Pearson correlation method was performed. As shown in **Figure 2**, PIVKA-II levels were positively correlated with AFP (r = 0.440; P <.0001; **Figure 2**, Image A) and GDF15 levels had positive correlations with AFP (r = 0.352; P <.0001) and PIVKA-II (r = 0.378; P <.0001; **Figure 2**, Images B and C).

Diagnostic Values of GDF15, AFP, and PIVKA-II to Differentiate Healthy Control Patients and Patients with CHB and LC from Patients with HCC

When the cutoff values of GDF15, AFP, and PIVKA-II were set as 817.46 pg/mL, 5.65 ng/mL, and 51.00 mAU/mL, respectively, for the detection of HCC, the area under the curve (AUC) of GDF15 was 0.693 (95% confidence interval [CI], 0.637–0.748), which was lower than that of AFP (0.826; 95% CI, 0.779–0.872) and PIVKA-II (0.935; 95% CI, 0.904–0.965; all P <.0001; **Figure 3**, Image A). When combined with AFP for the detection of HCC, the AUC of PIVKA-II increased slightly, with values of 0.937 (95% CI, 0.908–0.965) vs 0.935 (95% CI, 0.904–0.965; P >.05). When combined with GDF15 for the detection of HCC, the AUC of PIVKA-II increased slightly, with values of 0.937 (95% CI, 0.907–0.966) vs 0.935 (95% CI, 0.904–0.965; P >.05). When combined with GDF15 for the detection of HCC, the AUC of AFP decreased, with values of 0.801 (95% CI, 0.754–0.848) vs 0.826 (95% CI, 0.779–0.872; P >.05). When PIVKA-II was combined with AFP and GDF15 for the detection of HCC, the AUC of PIVKA-II decreased, with values of 0.931 (95% CI, 0.899–0.963) vs 0.935 (95% CI, 0.904–0.965; P >.05; **Figure 3**, Image B). Moreover, PIVKA-II had high sensitivity and specificity for distinguishing patients with HCC from both healthy control patients and patients with benign liver diseases, with values of 85.00% and 93.30%, respectively; and diagnostic sensitivity increased after the combined use of the 3 markers with a value of 87.60%, as shown in **Table 3**.

Diagnostic Values of GDF15, AFP, and PIVKA-II to Distinguish Between Patients with HCC and Patients with LC

Effective screening of patients with LC with a higher risk of liver cancer development is a key factor in improving the survival rate of patients with liver cancer but has long been a clinical problem. In this study, we therefore evaluated the potential of GDF15, AFP, and PIVKA-II as serum markers for differentiating HCC from LC. The ROC curve analyses indicated that compared with AFP and GDF15, PIVKA-II showed a higher AUC (0.910 vs 0.722 and 0.910 vs 0.593, respectively; all P < .0001; **Figure 4**, Image A). When combined with GDF15, PIVKA-II had the largest AUC and specificity: The values were 0.937% and 97.18%, respectively, but compared with other combinations, the difference was not statistically significant (P >.05; **Figure 4**, Image B). When GDF15 was combined with AFP, the diagnostic accuracy, sensitivity, and specificity of AFP were slightly improved (0.734 vs 0.722, 53.10 vs 48.67, 88.73 vs 84.51, respectively; P >.05). Other detailed comparisons are shown in **Table 4**.

DOI: 10.1093/labmed/lmaa089

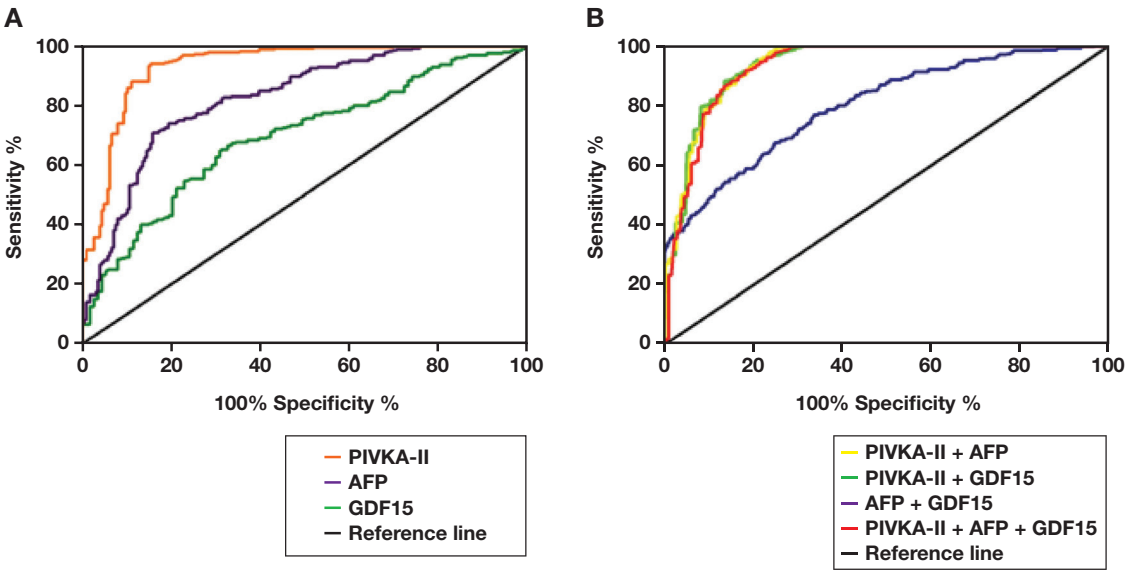

Figure 3

ROC curves of PIVKA-II, AFP, and GDF15 **(A)** and their combinations **(B)** in patients with HCC. Healthy patients, patients with CHB, and patients with LC were used as control patients (see Reference line).

Table 3. ROC Curve Analyses with Utility of Single or Combined Markers to Distinguish Patients with HCC from Healthy Control Patients and Patients with CHB and LC

Marker	Cutoff	SEN (%)	SPE (%)	AUC
PIVKA-II (mAU/mL)	51.00	85.00	93.30	0.935 (0.904–0.965)
AFP (ng/mL)	5.65	84.10	70.90	0.826 (0.779–0.872)[a]
GDF15 (pg/mL)	817.46	67.30	66.70	0.693 (0.637–0.748)[a,b]
PIVKA-II + AFP	NA	85.80	89.50	0.937 (0.908–0.965)[b,c]
PIVKA-II + GDF15	NA	85.00	91.60	0.937 (0.907–0.966)[b,c]
AFP + GDF15	NA	71.70	74.00	0.801 (0.754–0.848)[a,c,d,e]
PIVKA-II + AFP + GDF15	NA	87.60	87.40	0.931 (0.899–0.963)[b,c,f]

AFP, alpha-fetoprotein; AUC, area under the curve; CHB, chronic hepatitis B; GDF15, growth differentiation factor 15; HCC, hepatocellular carcinoma; LC, liver cirrhosis; NA, not applicable; PIVKA-II, protein induced by vitamin K absence -II; ROC, receiver operating characteristic; SEN, sensitivity; SPE, specificity.
[a]*Compared with PIVKA-II, P <.0001.*
[b]*Compared with AFP, P <.0001.*
[c]*Compared with GDF15, P <.0001.*
[d]*Compared with PIVKA-II + AFP, P <.0001.*
[e]*Compared with PIVKA-II + GDF15, P <.0001.*
[f]*Compared with AFP + GDF15, P <.0001.*

Discussion

The complexity of the pathogenesis of HCC means that it is difficult to diagnose HCC with a single biomarker. Therefore, the combination of multiple biomarkers may be more meaningful for the detection of HCC. This study focused on the evaluation of the diagnostic values of GDF15, PIVKA-II, and AFP in patients with HCC and discussed the above 3 biomarkers in different stages and different liver function states of patients with HCC and their correlation. Results showed that when compared with values in all control patients or patients with LC, the diagnostic value of PIVKA-II was superior to that of GDF15 and AFP, and AFP was also superior to GDF15 in the detection of HCC. However, the combination of PIVKA-II with GDF15 could improve the diagnostic value for distinguishing between HCC and LC. In addition, noting that high levels of GDF15 may be associated with poor liver function, we found that serum GDF15, PIVKA-II, and AFP levels were significantly elevated in patients with advanced HCC.

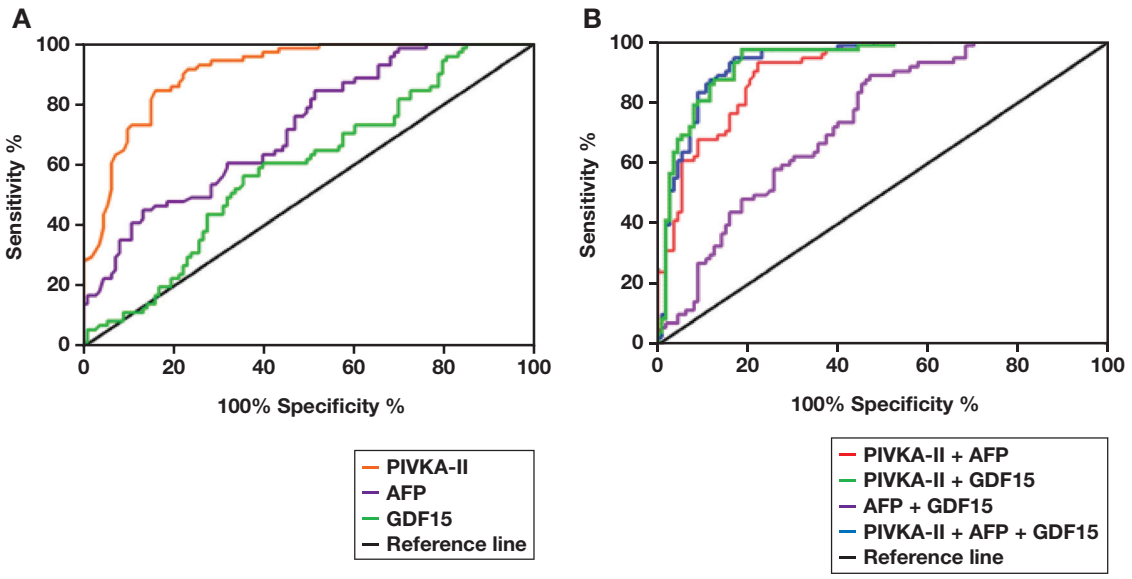

Figure 4

ROC curves of PIVKA-II, AFP, and GDF15 **(A)** and their combinations **(B)** in patients with HCC patients. Patients with LC were used as control patients (see Reference line).

Table 4. ROC Curve Analyses with Utility of Single or Combined Markers for Distinguishing Patients with HCC from Patients with LC

Marker	Cutoff	SEN (%)	SPE (%)	AUC
PIVKA-II (mAU/mL)	51.00	84.07	84.51	0.910 (0.859–0.947)
AFP (ng/mL)	89.40	48.67	84.51	0.722 (0.651–0.785)[a]
GDF15 (pg/mL)	1553.94	64.60	56.34	0.592 (0.517–0.633)[a,b]
PIVKA-II + AFP	NA	77.78	92.96	0.905 (0.853–0.943)[b,c]
PIVKA-II + GDF15	NA	81.42	97.18	0.937 (0.891–0.967)[b,c]
AFP + GDF15	NA	53.10	88.73	0.734 (0.664–796)[a,c,d,e]
PIVKA-II + AFP + GDF15	NA	83.19	94.37	0.936 (0.890–0.966)[b,c,f]

AFP, alpha-fetoprotein; AUC, area under the curve; GDF15, growth differentiation factor 15; HCC, hepatocellular carcinoma; LC, liver cirrhosis; NA, not applicable; PIVKA-II, protein induced by vitamin K absence -II; ROC, receiver operating characteristic; SEN, sensitivity; SPE, specificity.
[a]*Compared with PIVKA-II, P <.0001.*
[b]*Compared with AFP, P <.0001.*
[c]*Compared with GDF15, P <.0001.*
[d]*Compared with PIVKA-II + AFP, P <.0001.*
[e]*Compared with PIVKA-II + GDF15, P <.0001.*
[f]*Compared with AFP + GDF15, P <.0001.*

Finally, Pearson correlation analysis showed that there was a significant positive correlation between these 3 markers.

Research has shown that GDF15 is a member of the transforming growth factor-β superfamily and is weakly expressed in physiological conditions except in pregnancy, but its level is significantly increased in response to inflammation and malignancy and is also stimulated by nonsteroidal anti-inflammatory drugs.[13-16] Previous work suggested that GDF15 was expressed in normal mouse liver tissues and hepatocytes and was rapidly up-regulated after various surgical operations and chemotherapy that caused acute liver injury and regeneration.[17] In addition, the expression of the GDF15 protein was significantly increased in HCC tissue compared with that in the adjacent peritumoral tissues and normal liver.[17] Mechanism research has shown that overexpression of GDF15 can promote the growth and metastasis of human liver cancer stem cell–like cells by activating the AKT/GSK-3β/β-catenin signaling pathway.[12] In addition, GDF15 can participate in the pathogenesis

DOI: 10.1093/labmed/lmaa089

of HCV by changing the signal transduction and proliferation of host cells through the AKT/GSK-3β/RAF signaling pathway.[18] Elevated GDF15 levels may be associated with the progression of chronic liver diseases caused by HCV, including hepatitis, LC, and HCC.[19]

In our study, we observed that there was a dynamic change in the progression of GDF15 levels in CHB to LC and HCC, which suggests that the continuous elevation of GDF15 may be a contributing factor to the deterioration of chronic liver disease and may be a good marker for disease progression. In addition, the levels of AFP and PIVKA-II were significantly increased in patients with HCC than in those with benign liver diseases and healthy control patients. Liu et al[17] and Halim et al[19] found that GDF15 levels were higher in patients with LC and HCC, which is consistent with our findings. Further analysis showed that patients with advanced HCC had higher GDF15, AFP, and PIVKA-II levels, suggesting that these 3 markers are linked to severe liver damage. Our study also found that GDF15 had a good correlation with AFP and PIVKA-II, suggesting that GDF15 may be a biomarker for the diagnosis of HCC.

Further, the analysis of ROC curves (patients with HCC vs all control patients) revealed that GDF15 had a certain application value for distinguishing healthy control patients and patients with benign liver disease from HCC with an AUC of 0.693, a lower value than AFP, with an AUC of 0.826, and PIVKA-II, with an AUC of 0.935. When combined with GDF15, the AUC of AFP was reduced (0.826 vs 0.801) and the AUC of PIVKA-II was increased (0.935 vs 0.937).

However, the results of Liu et al[17] are somewhat different from ours: They found that compared with any other condition (including healthy control patients, patients who were HBV or HCV carriers, and patients with LC), the diagnostic AUC of GDF15 was superior to that of AFP in diagnosing HCC (0.840 vs 0.760) and that when the 2 were combined, the AUC value reached 0.910. We speculated that the difference may result from the selection of control patients and population differences. In addition, when it was combined with PIVKA-II, the diagnostic value of AFP was elevated (0.826 vs 0.937). Notably, among the 3 diagnostic indicators, PIVKA-II had the best diagnostic effect alone, with a sensitivity of 85.00% and a specificity of 88.90% for the diagnosis of HCC. In addition, ROC curves were also drawn to compare patients with HCC with patients with cirrhosis, who had a higher risk of tumor progression; we found that the combination of GDF15 and PIVKA-II could increase the diagnostic efficacy and specificity of PIVKA-II, which further suggests that GDF15 has an application value of assisting in the detection of HBV-related HCC.

There are several limitations to this research. In our study population, patients with HCC had inevitable individual differences, resulting in a high variability in GDF15, AFP, and PIVKA-II levels. Moreover, it is impossible to determine cause-and-effect relationships or the direction of influence based on our findings. A larger patient sample and longitudinal studies are needed to confirm our findings.

Conclusion

We found that PIVKA-II, AFP, and GDF15 can be used as noninvasive biomarkers for the detection of HBV-associated HCC, but PIVKA-II showed higher diagnostic efficiency compared to GDF15 and AFP. Furthermore, PIVKA-II combined with GDF15 can maximize the diagnostic performance of HBV-associated HCC. **LM**

Acknowledgments

PZ, YL, JC, and DT proposed the concept of the work. JC carried out most of the experimental work and wrote the article. ZN, CX, and HL provided critical reviews to promote the manuscript. All authors read and approved the final manuscript. This research was supported by the National Natural Science Foundation of China (grant number 81773444) and the Hubei Natural Science Foundation of China (grant number 2019CFC846). This study was reviewed and approved by the Medical Ethics Review Committee of Renmin Hospital of Wuhan University. All participants approved and signed a written informed consent consistent with the policies of Renmin Hospital of Wuhan University Ethics Committee.

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DOI: 10.1093/labmed/lmaa089

Light Chain Predominant Intact Immunoglobulin Monoclonal Gammopathy Disorders: Shorter Survival in Light Chain Predominant Multiple Myelomas

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Laboratory Medicine 2021;52:390-398

DOI: 10.1093/labmed/lmaa057

ABSTRACT

Background: A proportion of intact immunoglobulin (Ig)–producing multiple myelomas (MMs) was observed to secrete much higher amounts of free light chains (LCs) than usual.

Objectives: To determine the change point between usual and LC-predominant intact Ig-secreting MMs and other monoclonal gammopathic manifestations and the biological significance of the observation.

Methods: We conducted retrospective examination of laboratory findings in 386 MM, 27 smoldering MM, and 179 monoclonal gammopathy of undetermined significance (MGUS) cases that secreted intact Igs. We recorded the highest levels of involved serum free LC, highest ratio of involved to uninvolved LC, highest concentration of involved LC per g of monoclonal Ig, and highest value for ratio of involved to uninvolved LCs divided by the monoclonal Ig concentration. Each data set was sorted into kappa- and lambda LC-associated lesions. Length of time, in months, between diagnosis and last contact with the patients having myeloma was recorded.

Results: Change point analysis of data revealed a subgroup of cases with distinctly higher levels of free LCs. In myelomas, including plasma cell leukemias, 16.4% of myelomas with kappa LCs and 22.3% of myelomas with lambda LCs, the LC secretion was distinctly higher than in the remaining cases, by a combination of 4 parameters, listed herein. Corresponding figures for smoldering myeloma (SMM) and monoclonal gammopathy of undetermined significance (MGUS) were 12.5, 27.3, 3.8, and 6.8, respectively. Ten of the 13 (77%) cases of plasma cell leukemia) and all cases of IgD myeloma ($n = 4$) showed excess secretion of serum free LCs. Among IgG and IgA myelomas, including plasma cell leukemias, the LC-predominant lesions had shorter survival, by an average of 22.5 months.

Conclusions: In total, 18.4% of MMs, including plasma cell leukemias, secrete distinctly higher amounts of serum free LCs than other intact Ig-secreting myelomas and confer significantly lower survival. Quantification of monoclonal serum free LCs may be useful in this subgroup in monitoring progress and potentially in ascertaining minimal residual disease. The findings also stress the need for separate criteria for kappa and lambda LC associated monoclonal gammopathic manifestations. The significantly shorter survival of patients with LC-predominant myelomas warrants consideration in prospective trials of treatments.

Abbreviations:

MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; MM, multiple myeloma; Igs, immunoglobulins; LCs, light chains; CRAB, hypercalcemia renal failure anemia and bone disease; IMWG, International Myeloma Working Group; SFLCA, serum free LC assay; SFLC, serum free light chain; SPEP, serum protein electrophoresis; SIFE, serum immunofixation electrophoresis; QUIET, quantification by ultrafiltration and immunofixation electrophoresis testing; LCPMM, LC-predominant intact Ig MM; MALDI-TOF, matrix-assisted laser desorption/ionization; CI, confidence interval; I/UI, ratio of involved to uninvolved serum free light-chain concentration; LC/g, concentration of SFLC in mg/L per g of monoclonal immunoglobulin in g/dL; ratio/g, ratio of involved to uninvolved SFLC concentrations divided by concentration of monoclonal immunoglobulin; Pos, positive; K, kappa; L, lambda

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Neoplastic monoclonal gammopathic manifestations consist of monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), and the malignant entity of multiple myeloma (MM; also known as plasma cell myeloma).[1,2] MM is the most common hematological malignant neoplasm in adults, other than the heterogeneous entity of non-Hodgkin lymphomas.[3] Approximately 85% of MM lesions secrete intact immunoglobulins (Igs) consisting of heavy chains and light chains (LCs). Approximately 13% of MMs secrete only LCs, and 2% to 3% may secrete more than 1 Ig type (ie, biclonal and triclonal), or not secrete any Igs (ie, nonsecretory MM).[4]

Diagnosis of MM is based on the laboratory finding of monoclonal Ig of greater than 3.0 g per dL, more than 10% plasma cells in bone marrow, and indicators of end organ damage

(ie, CRAB [hypercalcemia, renal failure, anemia, and bone disease] criteria). The International Myeloma Working Group (IMWG) has defined an additional entity of myeloma-defining condition based on concentration of serum free LCs: greater than 60% plasma cells in bone marrow and more than 1 bone lesion, as measured by MRI. The criterion of using serum free LCs stipulates concentration of involved LCs as greater than 100 mg per L and ratio of involved to uninvolved LC concentration of greater than 100. This criterion does not address differences in kappa and lambda LCs and has sensitivity of 16% in predicting progression of disease.[5–7]

The IMWG considers serum and urine protein electrophoresis and immunofixation electrophoresis-based detection of monoclonal Igs as the criterion standard in diagnosing MM and recommends measurement of serum free LCs.[5,6] The serum free LC assay (SFLCA) is useful in monitoring LC myelomas.[8]

The generally accepted reference range for kappa/lambda ratio of 0.26 to 1.65 has many false-positive and false-negative results.[4,9,10] Using normal kappa/lambda ratio as a criterion for stringent complete response to treatment for MM has been questioned due to a high rate of false-positive kappa-dominant abnormal kappa/lambda ratio, after autologous stem cell transplantation.[11] It has been observed[12,13] that excess free kappa LCs, polyclonal as well as monoclonal, are secreted in greater abundance than lambda LCs, and that this observation should be considered for criteria using serum free light chain (SFLC) concentrations.

In addition to the usual greater excess of kappa LC production in benign and malignant conditions, we observed that a subgroup of intact Ig-producing monoclonal gammopathic disorders associated with kappa and lambda LCs had a distinctly higher concentration of involved SFLCs. This retrospective observational study was conducted to ascertain whether objective diagnostic criteria could be developed for this subgroup of monoclonal gammopathic lesions and to ascertain the biological significance of variation in excess free monoclonal LC production in MMs.

Methods

This retrospective observational study was conducted at a 480-bed medical school–affiliated, tertiary-care medical center in the southeastern United States. The investigation was approved by the institutional review board.

As described in earlier publications,[4,8,10] serum protein electrophoresis (SPEP) and serum immunofixation electrophoresis (SIFE) were carried out using Helena Laboratories instruments and reagent kits. SFLCA was performed by using equipment and testing kits obtained from The Binding Site.

Medical records, including laboratory data, for patients receiving SPEP and/or SIFE were reviewed for the period of January 2010 through August 2020. All patients in whom an intact monoclonal IgG or IgA was documented were evaluated for SFLC, SPEP, SIFE, and SFLCA results. Patients in whom a measurable monoclonal Ig was not detected were excluded. (Such patients had been treated at other institutions and were referred for consideration of hematopoietic stem cell transplantation.) Concentration of IgA was substituted for monoclonal IgA in patients with the monoclonal band in the beta region when a peak was not quantifiable on SPEP, but monoclonal IgA was detectable by SIFE. Patients with IgD myelomas were evaluated separately, as were patients with plasma cell leukemias. IgM monoclonal lesions, nonsecretory lesions, and LC-only monoclonal gammopathic manifestations were not included.

Data from patients with IgG, as well as IgA MGUS, SMM, and MM, were segregated and further subdivided based on the involved LC type. The following 4 parameters were ascertained: highest levels of SFLC in mg per L, highest ratio of involved to uninvolved SFLCs, highest reading of SFLC per g of intact monoclonal Ig, and highest value of involved to uninvolved LC concentration ratio divided by the concentration of monoclonal Ig. All 4 parameters were sorted from smallest to largest reading and analyzed for an inflection or change point.

We performed change point analysis using the *R* software package ChangePoint. This package is based on a maximum-likelihood statistic comparing the likelihood with and without a change point in the sorted data for each variable.[14] The null hypothesis was that the data come from 1 exponential distribution. The alternative hypothesis was that there are 2 exponential distributions separated by the change point C. The value of C that maximized the likelihood ratio statistic was chosen. Patients with readings exceeding the change point for all 4 parameters were designated as having LC-predominant intact Ig monoclonal gammopathic manifestations.

DOI: 10.1093/labmed/lmaa057

The data from patients with IgG and IgA myelomas seen between January 2010 and August 2020, including those with plasma cell leukemias, were extracted for date of diagnosis and date of last contact with the patient. In the date of diagnosis category, we recorded the month and year of diagnosis. The date of last contact category included the lattermost of the following: date of death (when known), date of last visit to the hospital, date of last laboratory examination, and date of telephone contact by or on behalf of the patient. These dates were recorded as month and year.

The length of time, in months, between the date of diagnosis and last contact was recorded as *survival time*. The survival times between the 2 groups of usual and LC-predominant myelomas were compared via *t*-testing using a 2-tailed comparison with assumption of different variance in the 2 populations to arrive at a conservative result. To minimize the error in survival data, patients whose last date of contact was in 2020 were excluded, and the survival data between the usual and LC-predominant myelomas were compared, as described earlier herein.

Serum specimen from 1 patient, who met the criteria for having LC-predominant IgG kappa MM and who had an intact monoclonal Ig detectable 1 month earlier but did not reveal a monoclonal Ig on SIFE on a recent specimen, was processed using the QUIET (quantification by ultrafiltration and immunofixation electrophoresis testing) method for detection of monoclonal LCs.[15]

Briefly, the QUIET method consists of separating low-molecular-weight proteins in serum by filtering through a membrane with a nominal exclusion limit of 50 kDa. The filtrate was concentrated with Millipore urine concentrator and further concentrated by lyophilization. The dry residue was dissolved in water and tested using SIFE. The reconstituted material was concentrated approximately 20- to 40-fold from the original volume of serum. The SFLC levels for kappa and lambda LCs were 18.3 mg per L and 0.5 mg per L, respectively, in the specimen processed using QUIET.

At 1 month earlier, monoclonal IgG kappa had been detectable via SIFE and free kappa LC was present at a concentration of 17.2 mg per L. SFLC kappa was noted at 300.7 mg per L at the time of diagnosis, 6 months earlier. For control individuals, sera from 1 patient with IgA kappa MM in remission and 1 patient with polyclonal increase in Igs were processed in parallel. The patient with IgA kappa myeloma had been diagnosed 8 years earlier with a kappa

Table 1. Kappa and Lambda Serum Free LC-Specific Change Points in MGUS, SMM, and MM[a]

Variable	Change Points											
	Kappa						Lambda					
	N Value	Mean(95% CI)				Pos for All, No. (%)	*N* Value	Mean (95% CI)				Pos for All, No. (%)
		I/lU	SFLC, mg/L	LC/g	Ratio/g			I/lU	SFLC, mg/L	LC/g	Ratio/g	
MGUS	105	6.39 (5.03–9.36)	12.33 (8.57–17.61)	25.73 (16.46–31.58)	12.43 (8.9–15.07)	4 (3.8%)	74	2.85 (1.92–3.62)	5.89 (4.27–8.18)	25.66 (12.8 –42.41)	4.98 (4.28–8.76)	5 (6.8%)
SMM	16	18.04 (4.91–203.35)	60.28 (2.38–258.79)	47.09 (5.33–267.23)	26.67 (3.80–119.03)	2 (12.5%)	11	3.01 (1.55–92.65)	7.74 (1.11–29.76)	6.90 (0.40–30.60)	4.85 (0.39–9.15)	3 (27.3%)
MM	256	44.90 (27.25–53.79)	35.15 (31.33–45.06)	67.02 (37.18–254.97)	68.44 (40.32–76.29)	42 (16.4%)	130	44.87 (29.70–76.67)	36.75 (27.12–70.16)	43.5 (23.83–105.32)	51.61 (34.51–146.94)	29 (22.3%)

LC, light chain; MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering multiple myeloma; MM, multiple myeloma; CI, confidence interval; I/Ul, ratio of involved to uninvolved serum free light-chain concentration; SFLC, serum free light chain concentration; LC/g concentration of SFLC in mg/L per g of monoclonal immunoglobulin in g/dL, ratio/g, ratio of involved to uninvolved SFLC concentrations divided by concentration of monoclonal immunoglobulin; Pos, positive.

[a]*A total of 18.4% of the MM lesions meet all 4 criteria for designation as LC-predominant intact immunoglobulin MM (LCPMM).*

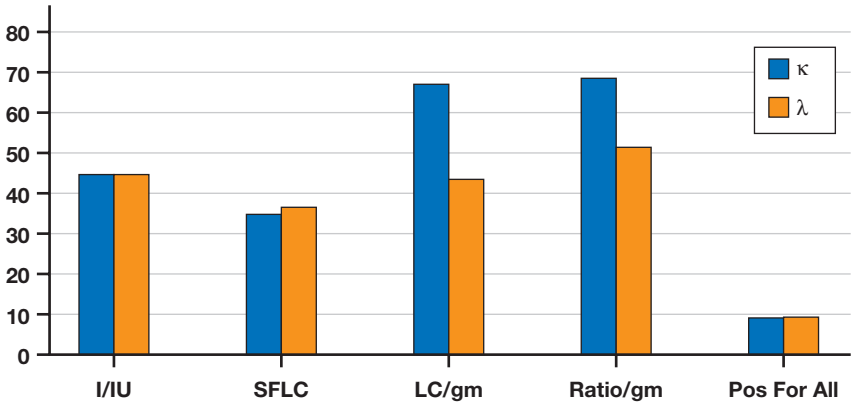

Figure 1

Results for the change point comparison between kappa- and lambda-chain–associated multiple myelomas (MMs) are shown graphically for the 4 parameters. The results of serum free light chain (LC)/g of monoclonal immunoglobulin (Ig) are higher in specimens from patients with kappa chain–associated MM. The same is true for the value of ratio of involved/uninvolved LC concentration divided by the concentration of monoclonal Ig. See Table 1 abbreviations for expansions of the abbreviations in Figure 1.

SFLC level at 738 mg per L. The patient had been in remission for the past 4 years. During the apparent remission period, his kappa LC levels varied from 1.7 mg per L to 52.2 mg per L. The serum specimen processed using QUIET had a kappa SFLC concentration of 8.7 mg per L The serum specimen from the patient with polyclonal hypergammaglobulinemia had a gammaglobulin level of 1.98 g per dL; levels of IgM, IgG, and IgA were 112 mg per dL, 1987 mg per dL, and 339 mg per dL, respectively. The kappa SFLC level was 8.73 mg per L.

Results

Requisite diagnostic criteria for MGUS, SMM, and MM were met, and laboratory data were available for 592 patients with IgG or IgA monoclonal gammopathic manifestations. IgG kappa or IgA kappa MM lesions were noted in 256 patients, and the corresponding figure for lambda LC–associated lesions was 130 patients. In this group of 386 patients with MM, a monoclonal Ig was present and quantifiable. The numbers for kappa and lambda MGUS lesions and kappa and lambda SMM lesions were 105, 74, 16, and 11, respectively (**Table 1**).

The change points for the 4 parameters for kappa and lambda LC-associated lesion of IgG and IgA type are shown in **Table 1**. The number and percentage of cases exceeding all 4 criteria are also noted in **Table 1**. Approximately 18.4% of the patients met the criteria for designation of LC-predominant intact Ig MM (LCPMM). Comparative change points for kappa and lambda LC-associated MM are shown graphically in **Figure 1**. Representative graphs and histograms illustrating the change point for the ratio of involved to uninvolved SFLC concentration divided by the monoclonal Ig concentration, in patients with MM, are shown in **Figure 2**.

Results of QUIET analysis for the 3 relevant patients are shown in **Figure 3**. The specimen in lane 1 was from a patient with LCP IgG kappa MM; no monoclonal Ig was detectable on SIFE in this specimen when processed via QUIET. Monoclonal kappa LC was detected on SIFE of concentrated ultrafiltrate of serum. The QUIET pattern from a patient with IgA kappa MM, who had been in remission for 4 years, showed polyclonal kappa LCs only (**Figure 3**, lane 2). The specimen from the patient with polyclonal hypergammaglobulinemia (lane 3), revealed polyclonal LC, as we had expected.

The results of 4 parameters for patients with plasma cell leukemia are shown in **Table 2**. All of the patients with IgD myelomas meet the criteria for classification as LC-predominant MM (LCPMM). Ten of 13 patients in the plasma cell leukemia group meet the criteria for being classified as having LCPMMs.

Lab Medicine 2021;52;390–398
DOI: 10.1093/labmed/lmaa057

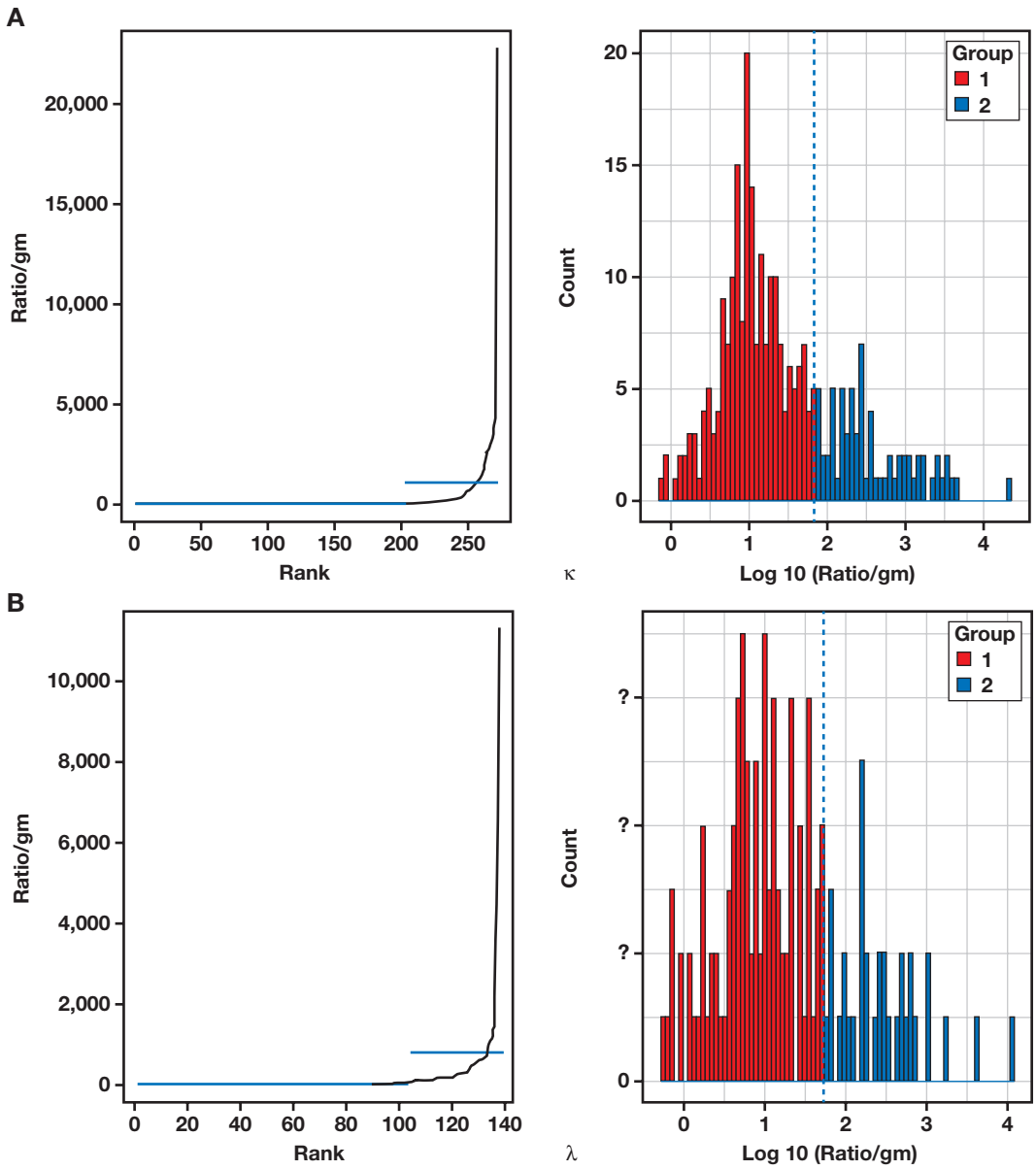

Figure 2

The graphs and histograms for the ratio of involved/uninvolved serum free light chain (SFLC) concentration divided by the concentration of monoclonal immunoglobulin (Ig: ratio/g) are shown for kappa- and lambda-chain–associated multiple myelomas (MMs). The panels on the left side show values for ratio of involved/uninvolved SFLC concentration divided by the concentration of monoclonal Ig (ratio/g) along the *y* axis. The numbers along the *x* axis are serial numbers for specimens, arranged in increasing order, for ratio/g. The change points are marked by the horizontal line at the lower right corner. The right-side panels display log to the base 10 transformed values for ratio/g along the *x* axis and number of observations for each value on the *y* axis. The change point is marked by the vertical dotted line.

The change points for MGUS and SMM, separated by kappa and lambda LCs, are shown in **Table 1**. The table also shows the number and proportion of cases in which all 4 criteria were exceeded in this group of nonmalignant monoclonal gammopathic manifestations.

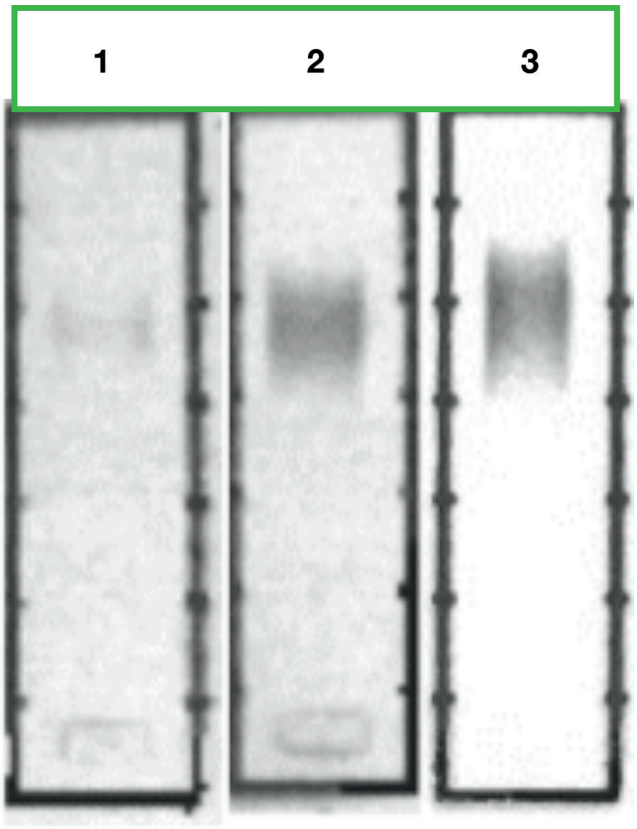

Figure 3

Serum immunofixation electrophoresis (SIFE) patterns of gels tested via the QUIET (quantification by ultrafiltration and immunofixation electrophoresis testing) method. All gels were stained for kappa light chains (LCs). Lane 1 represents concentrated ultrafiltrate of serum from a patient with immunoglobulin (Ig)G kappa LC-predominant intact immunoglobulin multiple myeloma (LCPMM) when no monoclonal IgG kappa was detectable via SIFE-detected patterns in serum. Note the monoclonal kappa LC band is noted. The faint staining is sometimes observed in monoclonal LCs due to variable reactivity of antisera with monoclonal LCs, as observed in an earlier study.[15] Lane 2 represents concentrated ultrafiltrate of serum from a patient with an LC-predominant IgA kappa multiple myeloma (MM) that had been in remission for 4 years at the time of specimen harvest. Note that there is only a polyclonal pattern. Lane 3 represents concentrated ultrafiltrate of serum from a patient with polyclonal hypergammaglobulinemia and shows a polyclonal pattern.

The survival data for patients with and without LCPMMs are shown in **Table 3**. On average, the LCPMMs had a shorter survival by 22.5 months. The averages, SDs, and ranges of survival for the 2 groups are listed in **Table 3**. The difference in survival between non-LCPMMs and LCPMMs was significant, at P <.001. Analysis of data by excluding cases of plasma cell leukemias did not change the comparative results materially (P <.001. When patients whose last date of contact was in 2020 were excluded, there remained 121 patients with usual myeloma and 43 with LCPMM. The survival in the LCPMM group was shorter by an average of 15.5 months, and the difference was significant (P = .03).

Discussion

Normal plasma cells, as well neoplastic ones, generally produce more LCs than heavy chains, and excess free LCs can be detected in serum and urine. There is considerable variability in the amount of excess free LCs produced by myeloma cells. Lambda LC-associated lesions produce lower amounts of excess free LCs than kappa LC-associated lesions.[12,13] In some MMs, there may be minimal production of excess free LCs, such that the kappa/lambda ratio in serum remains within normal limits.[4]

Lab Medicine 2021;52;390–398
DOI: 10.1093/labmed/lmaa057

Table 2. Values for the 4 Change Points in 13 Patients With Plasma Cell Leukemia and 4 Patients With IgD MM[a]

Ig Type	No.			
	I/UI	SFLC, mg/L	LC/g	Ratio/g
Plasma Cell Leukemia				
IgG K	2285.09	525.57	2627.85	11,425.43
IgA K	1555.00	311.00	1295.83	6479.17
IgA L	718.60	618.00	2809.09	3266.38
IgA L	856.07	2611.00	842.26	276.15
IgA L	133.93	20.09	60.88	405.86
IgA L	20,046.67	3007.00	2484.62	22,587.41
IgA L	665.43	2734.90	3447.29	796.14
IgG L	1473.33	274.00	110.04	438.49
IgG L	603.24	102.55	104.51	475.06
IgA L	3600.00	612.00	6120.00	4250.00
IgG L	15,866.67	2380.00	1694.59	11,297.30
IgA L	16.25	65.00	49.62	12.40
IgG L	0.94	1.37	1.35	1.13
IgD MM				
L	10,215.52	5925.00	4717.35	4745.09
L	3238.3	5213.66	2660.03	1652.19
L	384.04	2346.46	872.29	154.85
K	21,631.58	4110.00	1818.58	9571.50

Ig, immunoglobulin; MM, multiple myeloma; I/UI, ratio of involved to uninvolved serum free light-chain concentration; SFLC, serum free light chain concentration; LC/g, concentration of SFLC in mg/L per g of monoclonal immunoglobulin in g/dL; ratio/g, ratio of involved to uninvolved SFLC concentrations divided by concentration of monoclonal immunoglobulin; K, kappa; L, lambda.

[a]*Ten of 13 patients with plasma cell leukemia and all patients with IgD MM met criteria for designation as having LC-predominant intact immunoglobulin MM (LCPMM).*

Table 3. Length of Survival in Patients with Non-LCPMM vs LCPMM[a]

Survival Variable	Non-LCPMM	LCPMM
Average (SD)	65.28 (52.28)	42.79 (41.06)
Range	2–277	1–232

LCPMM, light chain–predominant intact immunoglobulin multiple myeloma.

[a]*P <.001.*

Approximately 90% of lambda chain–associated MGUS lesions exhibit normal kappa/lambda ratio; the corresponding figure for kappa chain–associated MGUS is approximately 60%. There is a spectrum in the amounts of LC production by myeloma cells, varying from no detectable excess production of LCs to secretion of LCs only.[4] Within this spectrum, a novel entity of intact Ig neoplastic lesions with greater excess of LC production, (ie, LC-predominant intact Ig MM [LCPMM]), is described herein.

Patients with LCPMMs have been shown to have significantly shorter survival, by nearly 2 years. A similar observation by Avivi et al[16] indicates that patients with high levels of SFLCs may have a worse prognosis. Monitoring the course of disease in this subgroup may benefit from measuring levels of monoclonal SFLCs. We emphasize that the SFLCA measures total serum free LCs, not monoclonal LCs, specifically. The LCPMM subgroup would be suitable for adoption of an assay for quantitative measurement of monoclonal SFLCs.[15,17] As demonstrated in **Figure 3**, patients with LCPMM may exhibit monoclonal serum free LCs via the QUIET assay when, after treatment, intact monoclonal Igs are no longer detectable by SIFE, thus demonstrating the presence of residual disease.

It is conceivable that relapse of disease in patients with LCPMM, after treatment, may be seen as LC escape. This appearance of LC only in serum may be due to marked excess of LCs that become detectable before intact monoclonal Ig becomes detectable. LC escape is a rare event, if it exists: we did not observe this phenomenon in the group of 386 patients, nor in those with IgD myelomas.

All of the IgD myelomas (n = 4), and 10 of the 13 IgG and IgA myelomas with plasma cell leukemia appear to be

LCPMMs and would be suitable for monitoring by QUIET or immunoaffinity concentration matrix-assisted laser desorption/ionization (MALDI-TOF) mass spectrometry, once the intact monoclonal Ig is no longer detectable after treatment.[15,17]

As has been reported earlier[4,10,13] and noted in this article as well, there is noticeable overproduction of kappa free LCs compared with lambda LCs in malignant and nonmalignant conditions. Thus, any criteria using SFLC as a parameter should have LC-specific values (eg, myeloma-defining condition) and may benefit from having kappa and lambda LC-specific trigger values for diagnosis. Similarly, the criteria developed by Avivi et al[16] may benefit from LC-specific criteria. A quantitative assay for monoclonal SFLC would be particularly relevant for this subgroup to detect residual or minimal residual disease.[15,17]

Four criteria were applied to designate a lesion as LCPMM because each individual criterion has interfering factors. SFLC levels are affected by renal clearance and variable tendency of the LCs to dimerize. Spuriously high SFLC levels in renal failure are partly corrected by using the ratio of involved to uninvolved LC concentration. For the same reason, SFLC and the ratio of involved to uninvolved LC ratio was normalized by the concentration of the monoclonal intact Ig. A much higher disparity in kappa- and lambda-chain parameters was noted[13] when the observations were limited to specimens with at least 0.3 g per dL of monoclonal Ig. The error rate in densitometric measurement of the monoclonal Ig at lower concentrations is higher.[18]

We expect that using all 4 criteria will provide a more conservative and reproducible identification of the LCPMMs. Of the 4 parameters applied in the experiments we describe herein , SFLC per g of monoclonal Ig appears to be the most pertinent. It is plausible that further study of similar patients may allow a single measure of SFLC per g of monoclonal Ig as the diagnostic criterion, albeit with separate decision points for kappa and lambda lesions.

This study has the usual drawbacks of an observational retrospective investigation. The suggestion for monitoring for monoclonal serum LCs via QUIET, after treatment and when intact monoclonal Ig is no longer detectable, is based on a single observation, albeit with support from an earlier publication.[15]

Estimation of survival was the most troublesome of the measurements because a definitive date of death could not be ascertained for many patients. As a result, we substituted the date of last contact for this measurement. Some patients were known to be alive at the conclusion of the study. The last shortcoming was addressed, partly, by excluding patients with the last date of contact in 2020. Thus, "survival" is an incomplete ascertainment in this study. We presume that the inaccurate ascertainment of length of survival is equally inaccurate in the 2 groups. The difference in "survival" of nearly 2 years between the 2 groups is no only highly significant statistically but is also clinically meaningful. This finding, although preliminary, provides a strong argument for identifying the patients with LCPMM in prospective trials of treatment.

Also, it may be pertinent to more closely monitor LC-predominant premalignant lesions of MGUS and SMM to identify patients at risk of renal damage and/or amyloidosis. Early treatment in such patients may be warranted if borne out by the results of controlled trials.

The reasons for shorter survival in this novel entity of LCPMMs could not be ascertained; however, there appeared to be a greater prevalence of renal failure in patients with LCPMM. The results of prospective studies may reveal a greater risk of renal disease and amyloidosis in the LCPMM group.

In summary, we have identified a novel entity of LCPMM. Approximately 18% of the MM lesions that produce intact Igs secrete much higher amounts of free LCs and confer significantly shorter survival rates on the affected patients. **LM**

Acknowledgements

We thank Roni J. Bollag, MD, PhD, for his expert professional advice.

Personal and Professional Conflicts of Interest

Dr Singh serves as a consultant to Diazyme Laboratories Inc. and is a member of the Medical Advisory Board for HealthTap. Dr Singh has applied for a United States patent for the QUIET method.

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Case Study

Hemolytic Disease of the Fetus and Newborn Caused by Maternal Autoantibody with Mimicking Anti-E Specificity

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Laboratory Medicine 2021;52:399-402

DOI: 10.1093/labmed/lmaa096

ABSTRACT

Objective: There are few reports of hemolytic disease of the fetus and newborn (HDFN) caused by maternal autoantibodies.

Methods: We describe the case of a pregnant patient aged 26 years with systemic lupus erythematosus without any transfusion history who developed autoantibody with mimicking anti-E specificity. Her newborn developed HDFN caused by the maternal autoantibody.

Results: The clinical symptoms of the newborn were not serious. After bilirubin light phototherapy and other symptomatic supportive treatment, the baby was discharged with a good prognosis.

Conclusion: This is the first reported case of HDFN caused by maternal autoantibody with mimicking anti-E specificity. However, the real antigenic target of the autoantibody was not clear.

Keywords: hemolytic disease of the fetus and newborn, autoantibodies, mimicking, anti-E

Patient History

Hemolytic disease of the fetus and newborn (HDFN) is a form of immunological hemolytic disease caused by maternal–fetal blood type incompatibility. The common cause involves IgG alloantibodies, which can cross the placental barrier. Only a few patients with cases caused by maternal autoantibodies have been reported. Herein, we report a case of a patient with HDFN caused by maternal autoantibody with mimicking anti-E specificity. On September 30, 2019, a 26 year old, 37 gestational weeks, G3P0Ab2 pregnant patient presented to our emergency department complaining of abdominal pain that had lasted for >3 hours, having had 2 miscarriages of unknown causes but associated with a history of systemic lupus erythematosus (SLE) for >3 years and undergoing treatment using long-term oral administration of prednisone and hydroxychloroquine.

Abbreviations:

HDFN, hemolytic disease of the fetus and newborn; SLE, systemic lupus erythematosus; DAT, direct antiglobulin test; PLT, platelet; RBCs, red blood cells; NT, not tested; W, weak; MC, mother's cell; NC, neonatal cell; TF-History, transfusion history; NM, not mentioned; GA, gestational age; POS, positive; NEG, negative; IAT, indirect antiglobulin test; PT, phototherapy; Hb, hemoglobin; TB, total bilirubin; SA, severe anemia; NP, neonatal pneumonia; RF, respiratory failure; HB, hyperbilirubinemia; Ex-TF, exchange transfusion; IVIG, intravenous immunoglobin; ALB, albumin.

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Clinical and Laboratory Information

During this third pregnancy, the mother had developed thrombocytopenia with a minimum of 35×10^9/L platelets (PLT; normal, $100–450\times10^9$/L), which was considered a complication of her SLE. She had no symptoms or transfusion

history. After admission, her hemoglobin level was 12.9 g/dL (normal, 11–15 g/dL) and her PLT count was 56×10^9/L. Serological results showed that her blood type was AB, her Rh phenotype was DccEE, and her direct antiglobulin test (DAT) was positive (4+); her antibody screening was I (0), II (2+), and III (0). Antibody identification of both the plasma and eluate showed IgG anti-E antibodies plus a positive autocontrol. Repeat serologic testing with the patient's adsorbed plasma showed the removal of apparent anti-E reactivity with either E-antigen-positive or E-antigen-negative red blood cells (RBCs). Adsorption of the plasma was performed based on the method described by Dwyre et al.[1] On the basis of her laboratory results and clinical history, the IgG anti-E was considered to be an autoantibody with mimicking anti-E specificity. Considering her thrombocytopenia, 1 apheresis PLT unit was transfused at the start of the second stage of labor, and the transfusion proceeded smoothly. Then she was transferred to deliver her baby right away. No other treatment was given in the emergency department. Her PLT count recovered to 86×10^9/L before discharge.

A healthy male baby was delivered at 37 weeks of gestation, weighing 2430 g. The Apgar scores were all 10 points for 1, 5, and 10 minutes. The baby was admitted to our neonatology department because of progressive cutaneous jaundice within 24 hours after birth. His hemoglobin level was 16.3 g/dL (normal, 17–21 g/dL), his reticulocyte count was 0.1698×10^{12}/L (normal, $0.024–0.084\times10^{12}$/L), total bilirubin was 16.81 mg/dL (normal, 0.58–11.11 mg/dL), and indirect bilirubin was 16.29 mg/dL (normal, 0.58–10.53 mg/dL). Considering the mimicking antibody detected in the maternal plasma and the potential blood needs of the baby, we collected the baby's umbilical cord blood for serological tests. The results showed that his neonatal blood type was B and the Rh phenotype was DCcEE; the DAT was positive (1+), and the antibody screening was I (0), II (1+), and III (0). Antibody identification of both the plasma and eluate showed IgG anti-E antibodies.

Combined with the baby's anemia, jaundice, and laboratory results, the diagnosis of HDFN was confirmed, and the cause was the transfer of the maternal autoantibody with mimicking anti-E specificity. A 16-cell antibody identification panel (Sanquin Reagents B.V., Amsterdam, Netherlands) was used, and the remaining serological determinations were performed using gel testing according to the manufacturer's instructions (Diagnostic Grifols, S.A., Barcelona, Spain). The serological results of the mother and her baby during hospitalization are summarized in **Table 1**.

Patient Follow-Up

Bilirubin light phototherapy and other symptomatic support treatment were given to the baby. Three days later, the jaundice subsided and the total bilirubin concentration decreased to 5.0 mg/dL. The baby was discharged with a good prognosis.

Discussion

Autoantibodies refer to those antibodies that act against one's own tissues, organs, cells, and cell components. When the body experiences immune system dysfunction because of autoimmune or other diseases, it may produce autoantibodies. According to Hoppe et al,[2] the chance of pregnant patients developing autoantibodies is at least 4 times higher than in nonpregnant peers. This process is usually caused by underlying diseases, immune system dysfunction during pregnancy, or other stimuli. In most patients, RBC autoantibodies react with all RBCs (ie, panreactive). Infrequently, in some patients the autoantibodies do have apparent specificity and do not maintain specificity after adsorption with antigen-negative and antigen-positive cells. These autoantibodies are said to be antibodies with mimicking specificity that were first described as "wrong antibodies," usually directed against Rh antigens (e, E, and c) although their true specificity is mostly anti-Hr or anti-Hr0.[3] In our patient, the mimicking specificity was confirmed because (1) antibody reactivity was consistent with anti-E, (2) the patient's RBCs were positive for the E-antigen, and (3) adsorbed serum (with either E-antigen-positive or E-antigen-negative RBCs) did not retain the anti-E activity. However, the real antigenic target of the autoantibody was not clear.

We searched PubMed and three Chinese language databases. To date, there are only 6 reports referring to HDFN caused by maternal autoantibodies. Of these, 3 involved nonspecific autoantibodies alone, 1 concerned a nonspecific autoantibody accompanied with an anti-E alloantibody, and the other 2 involved anti-M and anti-LW autoantibodies, respectively. All the relevant reports are summarized in **Table 2**. Studies have shown that maternal autoantibodies can lead to HDFN with varying

Table 1. Results of Screening Tests and Antibody Identification for Pregnant Patient and Infant in Plasma and Eluate

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

MC, mother's cell; NC, neonatal cell; NT, not tested; W, weak.

DOI: 10.1093/labmed/lmaa096

Table 2. Review of Published HDFN Literature Caused by Autoantibody

Case	Antibody	Pregnant Woman							GA	Infant						
		Age	Gravida-Para	TF-History	ABO	Rh	DAT	IAT	Titer		ABO	Rh	DAT	IAT	Hb g/dL)	TB mg/dL)	Major Complications	Management	Outcome
1[4]	Auto anti-LW	36	G2P1	NM	A	RhD (+)	POS	POS	NM	Term delivery	AB	RhD (+)	POS	NEG	NM	6.73	Jaundice	PT	Alive
2[5]	Nonspecific IgG autoantibody	25	G2P1	No	O	CcDee	POS (4+)	POS	IgM 4 IgG 256	NM	O	CcDee	POS (4+)	POS	NM	20.53	Jaundice	NM	NM
3[6]	Nonspecific IgG autoantibody	NM	G2P2	NM	B	RhD (+)	POS	POS	16	39+4	O	RhD (+)	POS	POS	14.8	16.41	Jaundice	PT	Alive
4[7]	Auto anti-M	35	G7P1	No	A	NM	NEG	POS	16	NM	NM	NM	NEG	POS	NM	NM	Jaundice	NM	NM
5[8]	Nonspecific IgG autoantibody/ alloantibody-e	21	G2P1	Yes	A	ccDEE	POS (4+)	POS[a]	64[b]	NM	O	CcDEe	POS (3+)	POS[c]	5	12.05	Jaundice/SA/ NP/RF/HB	TF/Ex-TF/PT/ IVIG/ALB	Alive
6[9]	Nonspecific IgG autoantibody	28	G1P0	No	B	RhD (+)	POS (4+)	POS (2+)	NM	NM	B	RhD (+)	POS (1+)	NEG	NM	NM	Jaundice	PT	Alive

ABO, blood type; ALB, albumin; anti-M, anti-M antibody; anti-LW, anti-LW antibody; DAT, direct antiglobulin test; Ex-TF, exchange transfusion; GA, gestational age; Hb, hemoglobin; HB, hyperbilirubinemia; IAT, indirect antiglobulin test; IVIG, intravenous immunoglobulin; NEG, negative; NM, not mentioned; NP, neonatal pneumonia; POS, positive; PT, phototherapy; RF, respiratory failure; Rh, Rh phenotype; SA, severe anemia; TF-History, transfusion history; TB, total bilirubin.

[a] The autoantibody DAT was positive (4+); the alloantibody-e DAT was positive (2+).

[b] The titer was tested at 4°C.

[c] The autoantibody IAT was positive (4+); the alloantibody-e IAT was positive (1+).

degrees. After active treatment, most of the babies born to mothers with these autoantibodies have a good prognosis.

This is the first reported case of a patient with HDFN caused by maternal autoantibody with mimicking anti-E specificity. It reminds clinicians to pay more attention to such a potential cause of HDFN. **LM**

Conflict of interest

The authors declare that there is no conflicts of interest.

Acknowledgments

We extend sincere gratitude and thanks to the staff of the Department of Laboratory Medicine, West China Second University Hospital, Sichuan University, China, and the investigators of the Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education. We also acknowledge with deep gratitude the assistance of James Cummins, PhD, from Liwen Bianji, Edanz Editing China, for editing the English text of a draft of this manuscript.

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Daratumumab Interference in Flow Cytometry Producing a False Kappa Light Chain Restriction in Plasma Cells

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Laboratory Medicine 2021;52:403-409

DOI: 10.1093/labmed/lmaa107

ABSTRACT

False kappa light chain restriction on hematogones (normal B-lineage precursors) has been described in patients on the therapeutic anti-CD38 monoclonal antibody daratumumab. In this article, we present a novel case report of pseudo-kappa light chain restriction on lambda-restricted neoplastic plasma cells in a patient with progressive plasma cell myeloma while on daratumumab. Flow cytometric technologists and pathologists need to be aware of this potential diagnostic pitfall.

Keywords: hematopathology, hematology, plasma cell myeloma, daratumumab, flow cytometry, pseudo-kappa restriction

Daratumumab, a therapeutic anti-CD38 IgG kappa monoclonal antibody, is becoming increasingly more common in the treatment of plasma cell myeloma. Such therapy can interfere with the detection of neoplastic cells because plasma cells are commonly identified in flow cytometry by bright CD38 and/or CD138 expression.[1] Pseudo-kappa restriction of early-stage hematogones has been reported in patients on daratumumab but has not been reported on malignant plasma cells.[2] In this case report, we present a patient with a history of lambda-restricted plasma cell myeloma with daratumumab interference producing false kappa light chain restriction in neoplastic plasma cells by flow cytometry.

Clinical History

A female patient aged 48 years was diagnosed with plasma cell myeloma 2 years ago after presenting with anemia. At initial presentation, she was found to have an IgG lambda monoclonal gammopathy with a monoclonal protein of 4.2 g/dL and a kappa-to-lambda free light chain ratio of 0.01:1. On skeletal survey there were numerous lytic lesions, a bone marrow biopsy revealed extensive involvement by lambda-restricted plasma cell myeloma, and flow cytometry identified a monotypic plasma cell population. Fluorescence in-situ hybridization on the bone marrow was positive for a gain of 1q, IGH/CCND1 (11;14) fusion, monosomy 13, and a gain of chromosome 17. The patient was initially treated with a bortezomib/lenalidomide/dexamethasone regimen for approximately 5 months. Because of lack of treatment response she was switched to a treatment including daratumumab, pomalidomide, and dexamethasone. The patient has been on maintenance daratumumab, pomalidomide, and dexamethasone for the past 5 months after a brief pause in treatment for hematopoietic stem cell collection. In anticipation of autologous stem cell transplant, a bone marrow biopsy was performed for restaging.

Flow cytometric analysis of the patient's bone marrow was performed using a 6-color FACSCanto II (Becton Dickinson Biosciences, San Jose, CA) analyzer using the 3-tube panel listed in **Table 1**. With the exception of Kappa (fluorescein isothiocyanate [FITC])/Lambda (phycoerythrin [PE]), a MultiMix from Dako (Carpinteria, CA), all antibodies were from Becton Dickinson Biosciences. The sample was prewashed 2 times with phosphate-buffered saline according to our normal procedure (the analysis was repeated

Abbreviation:

SPEP, serum protein electrophoresis.

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Table 1. The 3-Tube Panel Used for Flow Cytometric Analysis

	FITC	PE	PerCP-Cy 5.5	Pe-Cy 7	APC	APC-H7
Tube 1	Kappa (polyclonal)	Lambda (polyclonal)	CD5	CD10	CD19	CD45
Tube 2	CD38	CD56	CD138	CD3	CD117	CD45
Tube 3	Cytoplasmic kappa (polyclonal)	Cytoplasmic lambda (polyclonal)	CD138	CD20	CD38	CD45

APC, allophycocyanin; APC-H7, APC-cyanine conjugate; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP-Cy 5.5, peridinin chlorophyll protein complex-cyanine conjugate; PE-Cy7, PE-cyanine conjugate

after the sample was prewashed again 4 times, yielding no significantly different results). The first 2 tubes of the panel contained surface staining only and were processed with a stain-lyse method using a Beckman Coulter (Brea, California) Whole Blood Lyse Kit (Immuno-Lyse followed by fixative). The third tube of the panel contained surface and cytoplasmic staining and was processed using a Becton Dickinson Intrasure kit and FACSLyse reagent.

Representative flow cytometry scatterplots are shown in **Figure 1**. The plasma cell population represented approximately 5% of all events, excluding debris and doublets. The plasma cells were identified by CD138 expression. The plasma cells and all other cells in a broad mononuclear gate were negative for CD38, compatible with daratumumab therapy. The plasma cells showed variably dim CD45 expression and were positive for CD56 and surface and cytoplasmic kappa light chains. The population was CD117-negative. The surface kappa light chains were brightly positive, whereas the cytoplasmic kappa light chains were slightly dim compared to the background polytypic B-lymphocytes.

Serum protein electrophoresis and immunofixation were performed on the same day as the bone marrow collection and showed a clonal IgG lambda band representing 95% of the gamma region (**Figure 2**). A serum free light chain analysis revealed kappa light chains slightly below the normal range at 0.30 mg/dL, lambda light chains well above the normal range at 9.36 mg/dL, and a low kappa-to-lambda ratio of 0.03:1.

The bone marrow biopsy was processed for morphologic evaluation using standard methods. Representative images from the morphologic evaluation are shown in **Image 1**. A manual differential count performed on Wright-Giemsa stained marrow aspirate slides yielded 18% plasma cells. The hematoxylin-and-eosin stained bone marrow core biopsy showed a hypercellular marrow with trilineage hematopoiesis and sheets of plasma cells, constituting approximately 50% of the marrow cells. By immunohistochemical stains, the plasma cells were positive for CD138 and cyclinD1 and were negative for CD20. By in-situ hybridization, the plasma cells were lambda-restricted.

After the restaging bone marrow biopsy showed persistent plasma cell myeloma, the patient's treatment was modified to a regimen of carfilzomib, cyclophosphamide, and dexamethasone. A bone marrow biopsy performed at our institution 5 months after cessation of daratumumab therapy showed extensive involvement by a lambda-restricted plasma cell neoplasm, and flow cytometry identified a lambda-restricted plasma cell population (**Figure 3**).

Discussion

As more monoclonal antibody therapies are developed, there will be potential for testing interference in the diagnosis and monitoring of patients treated with these therapies. Daratumumab is an anti-CD38 IgG kappa monoclonal antibody that produces immune-mediated destruction of tumor cells expressing CD38 by apoptosis, phagocytosis, and other types of antibody-dependent cytotoxicity.[3] CD38 is expressed on T cells, B cells, natural killer cells, and monocyte subsets; on red blood cells and platelets; in nonhematopoietic tissues; and on plasma cells.[4] Direct antibody binding has led to problems in multiple areas of laboratory testing such as falsely positive indirect Coombs testing in transfusion workups, clonal IgG kappa bands seen in serum protein electrophoresis (SPEP), and identification of plasma cells by flow cytometry (typically using a gating strategy of CD38 + CD138+ events). In this case study, we present an example of daratumumab interference that not only changed the identification strategy

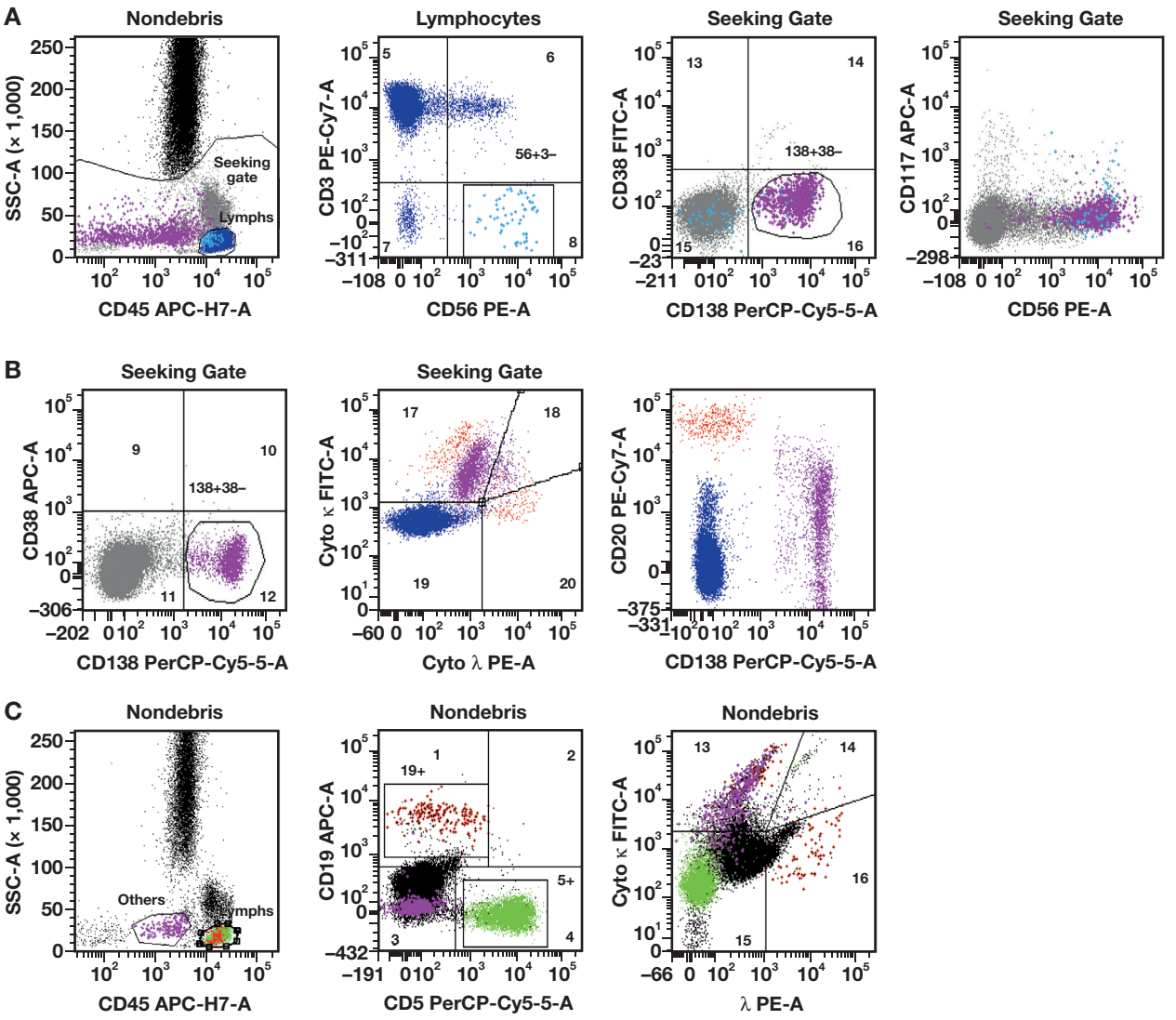

Figure 1

Representative flow cytometry scatterplots. **Row A** shows representative scatterplots from tube 2. In the CD45 by side-scatterplot showing cells after exclusion of doublets and debris, the plasma cells (purple) have low side-scatter and variable CD45 expression. Populations of lymphocytes (blue), monocytes, granulocytes, and erythroid precursors are also present. Natural killer cells (light blue) are identified in a CD3 vs CD56 plot of the lymphocyte gate. The last 2 plots show that the plasma cells are positive for CD138 and CD56 and are negative for CD38 and CD117. **Row B** shows representative scatterplots from tube 3. Plasma cells (purple) are identified by CD138 expression. The B cells (red), identified by CD20 expression, are polytypic. The plasma cell events appear kappa restricted, with slightly dimmer cytoplasmic kappa expression than the B cells. **Row C** shows representative scatterplots from tube 1. Plasma cells (purple) are identified by the characteristic positioning of the population in the CD45 by side-scatterplot. The B cells, identified by CD19 expression, are colored red, and T cells, identified by CD5, are colored light green. The plasma cell population has an expression of surface kappa light chains, with a brightness similar to the background polytypic B cells. Note that there were fewer total events acquired in tube 1 than in tubes 2 and 3, explaining the appearance of fewer plasma cells and fewer B-cell events.

but also seemed to produce a false kappa light chain restriction. False-positive surface kappa light chain restriction by flow cytometry has been reported in patients on alemtuzumab,[5] and pseudo-kappa restriction of early-stage (stage I/II) hematogones by flow cytometry has been reported in patients on daratumumab.[2] This is the first report of pseudo-kappa restriction of neoplastic plasma cells in a patient on daratumumab. Awareness of this potential pitfall

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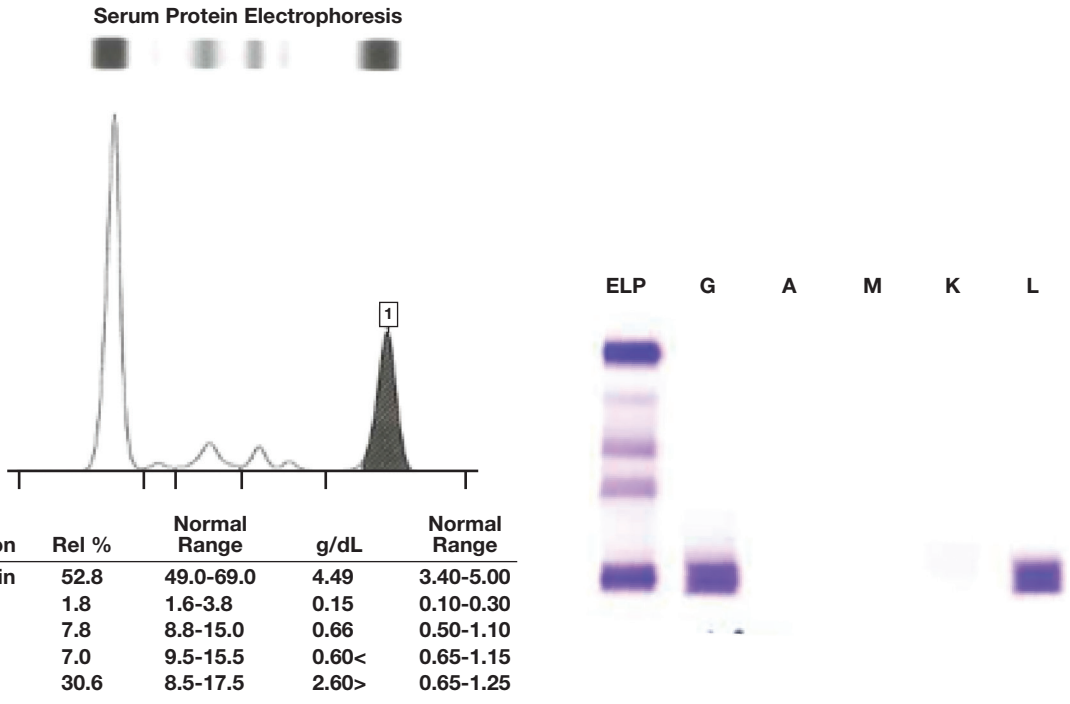

Fraction	Rel %	Normal Range	g/dL	Normal Range
Albumin	52.8	49.0-69.0	4.49	3.40-5.00
α1	1.8	1.6-3.8	0.15	0.10-0.30
α2	7.8	8.8-15.0	0.66	0.50-1.10
β	7.0	9.5-15.5	0.60<	0.65-1.15
γ	30.6	8.5-17.5	2.60>	0.65-1.25

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Serum protein electrophoresis. The results of serum protein electrophoresis and immunofixation performed the same day as the bone marrow biopsy are shown. An IgG lambda monoclonal protein was identified, representing 95% of the gamma region or 2.48 g/dL.

is important for proper interpretation of flow cytometry data and to avoid reporting inaccurate results.

For the technologist and hematopathologist performing the interpretation of the flow cytometry data, correlation with the clinical history of a lambda-restricted plasma cell myeloma was the first clue to a possible false kappa restriction. Although the patient could have undergone a class-switch among her clonal plasma cells, this phenomenon happens rarely and is more commonly seen after intensive chemotherapy.[6] Correlation with the results of other testing performed on specimens from the same patient on the same day, including SPEP and immunofixation and bone marrow morphologic evaluation, was vital. Most notably, on evaluation of the bone marrow core biopsy, the plasma cells were observed to be lambda-restricted using chromogenic in situ hybridization. This method is ultrasensitive and is based on messenger RNA rather than the antibodies themselves.[7] Note that although previous serum immunofixation studies had identified a very faint comigrating IgG kappa band in addition to the IgG lambda band, this band was not clearly shown in the current study. This potential SPEP artifact is known to occur with monoclonal antibody therapies, including daratumumab, and is typically seen as a small IgG kappa band in the slow gamma region.[8] The lack of a demonstrable comigrating IgG kappa band in the current study may result from the larger relative contribution of the monoclonal IgG lambda protein compared to prior SPEP studies.

When the discrepancy in light chain restriction from the reported history was identified in this patient, the technologist first carefully excluded specimen mixup and repeated the setup and analysis of the specimen. On repeat of the specimen preparation, 2 additional prewashes were performed, with no change in results (data not shown). At the time of this case study, the light chain antibodies used in our laboratory were a manufactured premixed cocktail, excluding incorrect pipetting of antibodies as an explanation. Additional potential troubleshooting steps include using a blocking serum or trying different kappa and lambda antibodies (monoclonal antibodies or those from a different vendor); we were not equipped to perform either of those strategies in our laboratory at the time of this study. Unfortunately, we did

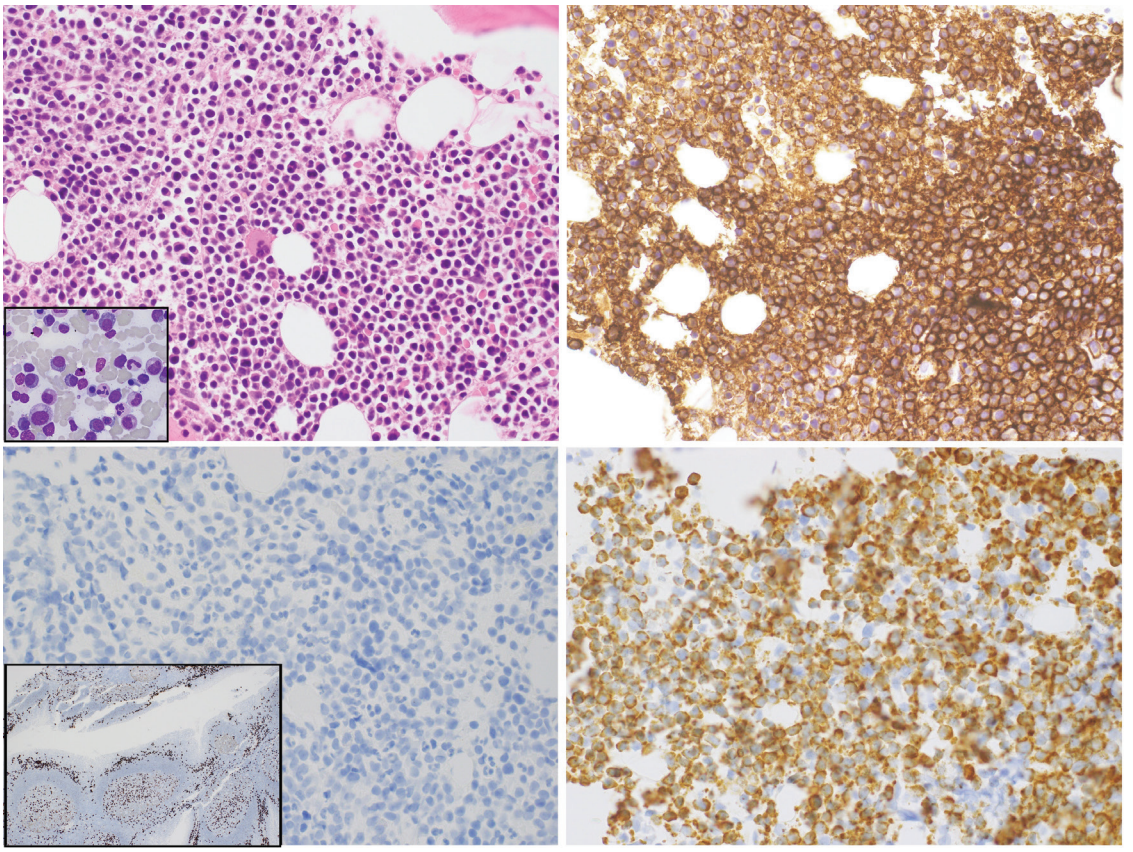

Image 1

Representative photomicrographs from bone marrow biopsy. **A,** Hematoxylin-and-eosin stained section of the bone marrow core showing sheets of abnormal plasma cells. Inset shows representative portion of Wright-Giemsa stained aspirate smear with frequent plasma cells. **B,** The plasma cells were positive for CD138 immunohistochemical stain performed on the core biopsy. By in-situ hybridization (RNAscope) performed on the core biopsy, the plasma cells were negative for kappa (**C**), inset showing on-slide positive control and positive for lambda (**D**). All images taken with 40× objective.

not have a significant population of hematogones or other known CD38-expressing cell types to further investigate the specificity of the kappa staining. Other than the clinical history, another clue that the findings represented false kappa restriction was the dimness of the cytoplasmic kappa expression, which was present at an intensity a log dimmer than typically seen on neoplastic plasma cells.

The presence of surface light chain pseudo-kappa restriction can be explained by the cross-reactivity of the fluorochrome-labeled anti-kappa antibody with daratumumab bound to CD38 on the surface of the neoplastic plasma cells. Applying the same explanation to cytoplasmic light chain staining, as seen in our patient, implies internalization of the daratumumab/CD38 complex into the neoplastic plasma cells. Internalization of the daratumumab/CD38 complex in multiple myeloma cell lines has been described[9] and we hypothesize this is the mechanism for the findings in this case report. The lack of lambda light chain staining in the plasma cells is likely because of the bound anti-CD38 monoclonal antibody preventing the fluorochrome-conjugated anti-lambda antibody from binding by a physical space–occupying mechanism.

Why was the pseudo-kappa restriction from daratumumab interference observed in this particular patient? Our flow cytometry laboratory has analyzed many specimens from other patients with multiple myeloma on daratumumab, and

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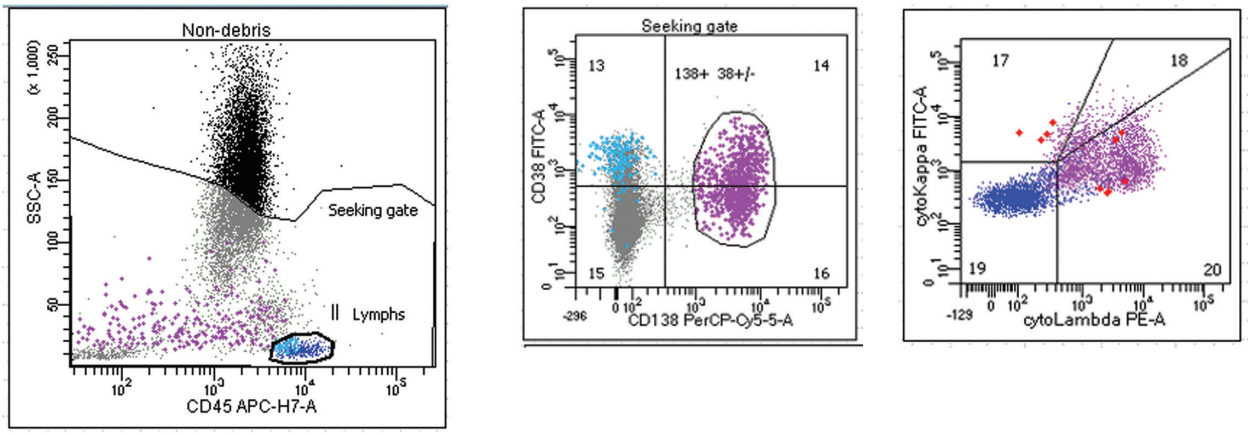

Figure 3

Representative flow cytometry scatterplots 5 months after cessation of daratumumab therapy. The first 2 scatterplots are from tube 2. The CD45 by side-scatter dot plot includes cells after exclusion of doublets and debris. The plasma cells are colored purple and the natural killer cells (identified on a CD3-by-CD56 plot, not shown) are colored light blue. Plasma cells are identified by CD138 expression and show dim CD38 expression and the natural killer cells are negative for CD138 and positive for CD38. The last scatterplot is from tube 3 and shows lymphocytes (identified by CD45 by side-scatter) and plasma cells (purple, identified by CD138 expression). The plasma cell population shows monotypic lambda light chain expression. The B cells (red, identified by CD20 expression) are polytypic.

this was the first time we saw this effect. Several factors may have contributed to this particularly striking effect. The patient had a high percentage of neoplastic plasma cells, suggesting that the daratumumab was not effective in clearing the neoplastic cells and enhancing the effect. The pseudo-kappa restriction may be difficult to identify if the burden of disease is low. The history of lambda-restricted neoplastic plasma cells also made the pseudo-kappa restriction more evident because it was a change from the history. It is conceivable that such a restriction could be interpreted as true light chain restriction in a patient with a history of kappa-restricted neoplastic plasma cells. Correlation with markers of aberrancy on plasma cells such as CD56, CD117, and weak CD81 would be especially important in such patients, along with corresponding bone marrow morphology. In addition, in theory the neoplastic plasma cells in this patient could have had an unusually high level of CD38 expression and/or an unusually high rate of daratumumab/CD38 internalization, leading to the artifactual staining with cytoplasmic light chain staining. A flow cytometry study performed on marrow aspirate from this patient 5 months after the cessation of daratumumab therapy was notable for plasma cells with monotypic cytoplasmic lambda light chain expression, providing further evidence for pseudo-kappa restriction in the presence of anti-CD38 monoclonal antibody therapy.

In summary, we present a case report of pseudo-kappa light chain restriction on neoplastic plasma cells in the context of daratumumab therapy. Technologists and pathologists need to be aware of this potential diagnostic pitfall. Correlation with clinical history, results of serum protein electrophoresis and immunofixation, and morphologic findings in the bone marrow are important. **LM**

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Daratumumab Interference in Flow Cytometry Producing a False Kappa Light Chain Restriction in Plasma Cells

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Laboratory Medicine 2021;52:403-409

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ABSTRACT

False kappa light chain restriction on hematogones (normal B-lineage precursors) has been described in patients on the therapeutic anti-CD38 monoclonal antibody daratumumab. In this article, we present a novel case report of pseudo-kappa light chain restriction on lambda-restricted neoplastic plasma cells in a patient with progressive plasma cell myeloma while on daratumumab. Flow cytometric technologists and pathologists need to be aware of this potential diagnostic pitfall.

Keywords: hematopathology, hematology, plasma cell myeloma, daratumumab, flow cytometry, pseudo-kappa restriction

Daratumumab, a therapeutic anti-CD38 IgG kappa monoclonal antibody, is becoming increasingly more common in the treatment of plasma cell myeloma. Such therapy can interfere with the detection of neoplastic cells because plasma cells are commonly identified in flow cytometry by bright CD38 and/or CD138 expression.[1] Pseudo-kappa restriction of early-stage hematogones has been reported in patients on daratumumab but has not been reported on malignant plasma cells.[2] In this case report, we present a patient with a history of lambda-restricted plasma cell myeloma with daratumumab interference producing false kappa light chain restriction in neoplastic plasma cells by flow cytometry.

Clinical History

A female patient aged 48 years was diagnosed with plasma cell myeloma 2 years ago after presenting with anemia. At initial presentation, she was found to have an IgG lambda monoclonal gammopathy with a monoclonal protein of 4.2 g/dL and a kappa-to-lambda free light chain ratio of 0.01:1. On skeletal survey there were numerous lytic lesions, a bone marrow biopsy revealed extensive involvement by lambda-restricted plasma cell myeloma, and flow cytometry identified a monotypic plasma cell population. Fluorescence in-situ hybridization on the bone marrow was positive for a gain of 1q, IGH/CCND1 (11;14) fusion, monosomy 13, and a gain of chromosome 17. The patient was initially treated with a bortezomib/lenalidomide/dexamethasone regimen for approximately 5 months. Because of lack of treatment response she was switched to a treatment including daratumumab, pomalidomide, and dexamethasone. The patient has been on maintenance daratumumab, pomalidomide, and dexamethasone for the past 5 months after a brief pause in treatment for hematopoietic stem cell collection. In anticipation of autologous stem cell transplant, a bone marrow biopsy was performed for restaging.

Flow cytometric analysis of the patient's bone marrow was performed using a 6-color FACSCanto II (Becton Dickinson Biosciences, San Jose, CA) analyzer using the 3-tube panel listed in **Table 1**. With the exception of Kappa (fluorescein isothiocyanate [FITC])/Lambda (phycoerythrin [PE]), a MultiMix from Dako (Carpinteria, CA), all antibodies were from Becton Dickinson Biosciences. The sample was prewashed 2 times with phosphate-buffered saline according to our normal procedure (the analysis was repeated

Abbreviation:

SPEP, serum protein electrophoresis.

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Table 1. The 3-Tube Panel Used for Flow Cytometric Analysis

	FITC	PE	PerCP-Cy 5.5	Pe-Cy 7	APC	APC-H7
Tube 1	Kappa (polyclonal)	Lambda (polyclonal)	CD5	CD10	CD19	CD45
Tube 2	CD38	CD56	CD138	CD3	CD117	CD45
Tube 3	Cytoplasmic kappa (polyclonal)	Cytoplasmic lambda (polyclonal)	CD138	CD20	CD38	CD45

APC, allophycocyanin; APC-H7, APC-cyanine conjugate; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP-Cy 5.5, peridinin chlorophyll protein complex-cyanine conjugate; PE-Cy7, PE-cyanine conjugate

after the sample was prewashed again 4 times, yielding no significantly different results). The first 2 tubes of the panel contained surface staining only and were processed with a stain-lyse method using a Beckman Coulter (Brea, California) Whole Blood Lyse Kit (Immuno-Lyse followed by fixative). The third tube of the panel contained surface and cytoplasmic staining and was processed using a Becton Dickinson Intrasure kit and FACSLyse reagent.

Representative flow cytometry scatterplots are shown in **Figure 1**. The plasma cell population represented approximately 5% of all events, excluding debris and doublets. The plasma cells were identified by CD138 expression. The plasma cells and all other cells in a broad mononuclear gate were negative for CD38, compatible with daratumumab therapy. The plasma cells showed variably dim CD45 expression and were positive for CD56 and surface and cytoplasmic kappa light chains. The population was CD117-negative. The surface kappa light chains were brightly positive, whereas the cytoplasmic kappa light chains were slightly dim compared to the background polytypic B-lymphocytes.

Serum protein electrophoresis and immunofixation were performed on the same day as the bone marrow collection and showed a clonal IgG lambda band representing 95% of the gamma region (**Figure 2**). A serum free light chain analysis revealed kappa light chains slightly below the normal range at 0.30 mg/dL, lambda light chains well above the normal range at 9.36 mg/dL, and a low kappa-to-lambda ratio of 0.03:1.

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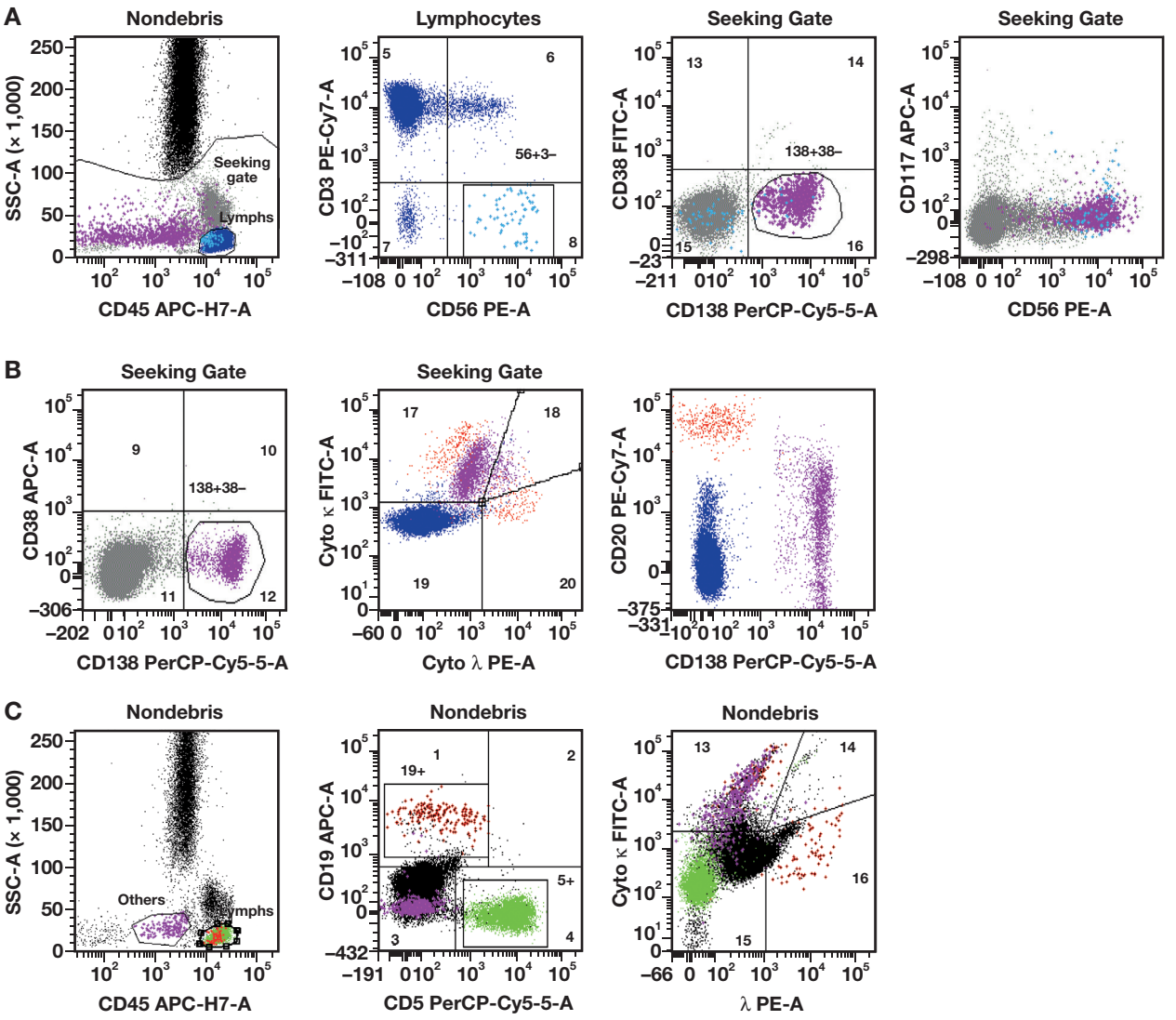

Figure 1

Representative flow cytometry scatterplots. **Row A** shows representative scatterplots from tube 2. In the CD45 by side-scatterplot showing cells after exclusion of doublets and debris, the plasma cells (purple) have low side-scatter and variable CD45 expression. Populations of lymphocytes (blue), monocytes, granulocytes, and erythroid precursors are also present. Natural killer cells (light blue) are identified in a CD3 vs CD56 plot of the lymphocyte gate. The last 2 plots show that the plasma cells are positive for CD138 and CD56 and are negative for CD38 and CD117. **Row B** shows representative scatterplots from tube 3. Plasma cells (purple) are identified by CD138 expression. The B cells (red), identified by CD20 expression, are polytypic. The plasma cell events appear kappa restricted, with slightly dimmer cytoplasmic kappa expression than the B cells. **Row C** shows representative scatterplots from tube 1. Plasma cells (purple) are identified by the characteristic positioning of the population in the CD45 by side-scatterplot. The B cells, identified by CD19 expression, are colored red, and T cells, identified by CD5, are colored light green. The plasma cell population has an expression of surface kappa light chains, with a brightness similar to the background polytypic B cells. Note that there were fewer total events acquired in tube 1 than in tubes 2 and 3, explaining the appearance of fewer plasma cells and fewer B-cell events.

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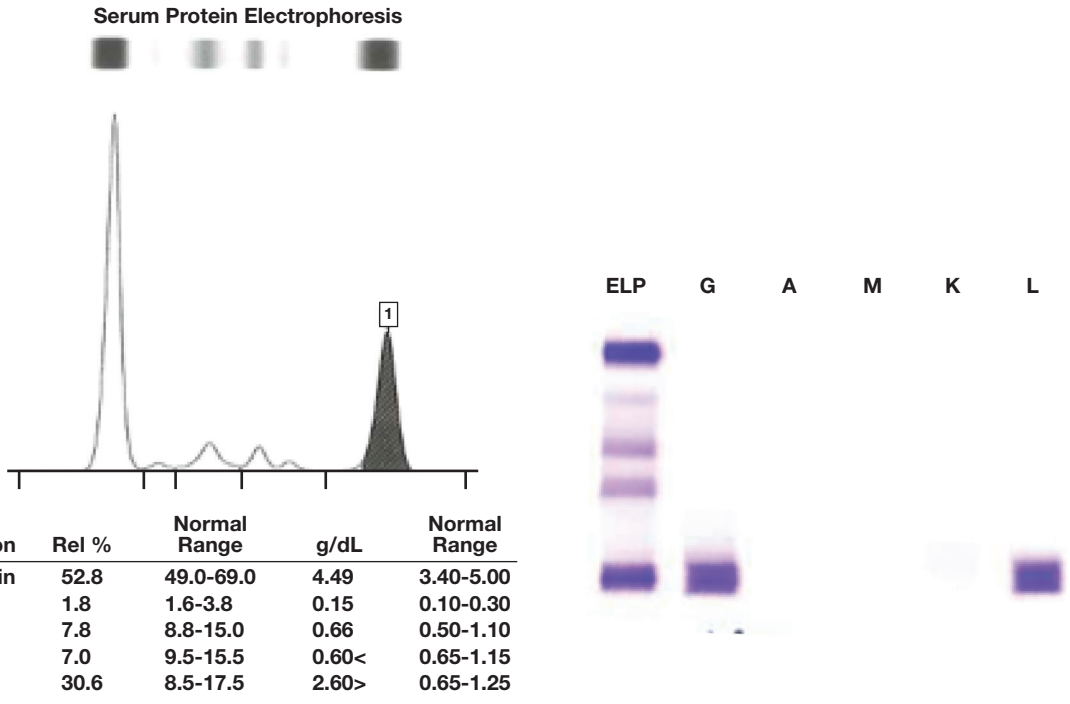

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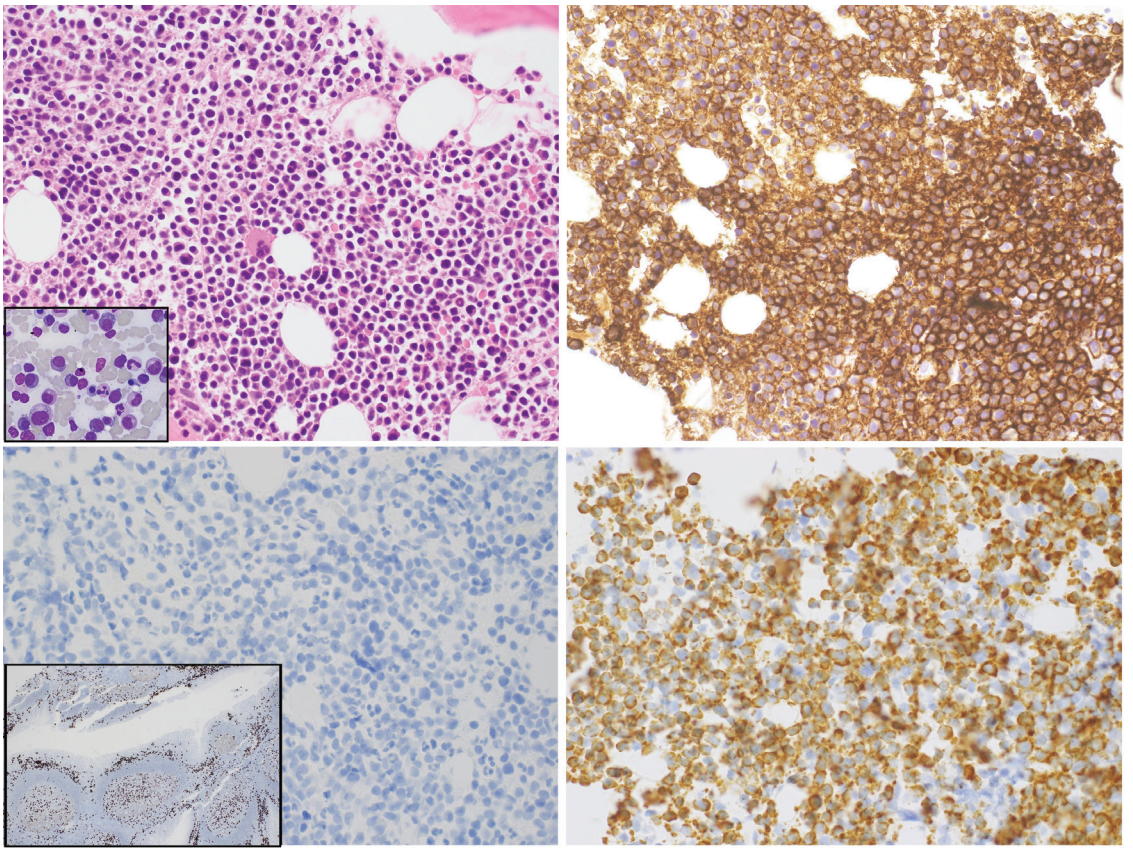

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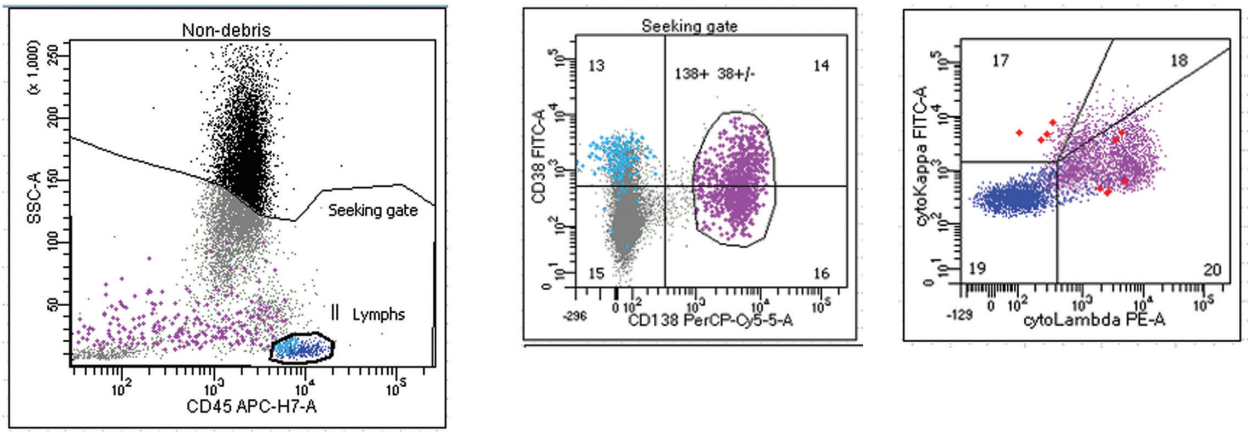

Figure 3

Representative flow cytometry scatterplots 5 months after cessation of daratumumab therapy. The first 2 scatterplots are from tube 2. The CD45 by side-scatter dot plot includes cells after exclusion of doublets and debris. The plasma cells are colored purple and the natural killer cells (identified on a CD3-by-CD56 plot, not shown) are colored light blue. Plasma cells are identified by CD138 expression and show dim CD38 expression and the natural killer cells are negative for CD138 and positive for CD38. The last scatterplot is from tube 3 and shows lymphocytes (identified by CD45 by side-scatter) and plasma cells (purple, identified by CD138 expression). The plasma cell population shows monotypic lambda light chain expression. The B cells (red, identified by CD20 expression) are polytypic.

this was the first time we saw this effect. Several factors may have contributed to this particularly striking effect. The patient had a high percentage of neoplastic plasma cells, suggesting that the daratumumab was not effective in clearing the neoplastic cells and enhancing the effect. The pseudo-kappa restriction may be difficult to identify if the burden of disease is low. The history of lambda-restricted neoplastic plasma cells also made the pseudo-kappa restriction more evident because it was a change from the history. It is conceivable that such a restriction could be interpreted as true light chain restriction in a patient with a history of kappa-restricted neoplastic plasma cells. Correlation with markers of aberrancy on plasma cells such as CD56, CD117, and weak CD81 would be especially important in such patients, along with corresponding bone marrow morphology. In addition, in theory the neoplastic plasma cells in this patient could have had an unusually high level of CD38 expression and/or an unusually high rate of daratumumab/CD38 internalization, leading to the artifactual staining with cytoplasmic light chain staining. A flow cytometry study performed on marrow aspirate from this patient 5 months after the cessation of daratumumab therapy was notable for plasma cells with monotypic cytoplasmic lambda light chain expression, providing further evidence for pseudo-kappa restriction in the presence of anti-CD38 monoclonal antibody therapy.

In summary, we present a case report of pseudo-kappa light chain restriction on neoplastic plasma cells in the context of daratumumab therapy. Technologists and pathologists need to be aware of this potential diagnostic pitfall. Correlation with clinical history, results of serum protein electrophoresis and immunofixation, and morphologic findings in the bone marrow are important. **LM**

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DOI: 10.1093/labmed/lmaa107

Special Report

The Impact of Mass Spectrometry on Patients' Medical and Nonmedical Lives

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Laboratory Medicine 2021;52:e58-e65

DOI: 10.1093/labmed/lmaa083

ABSTRACT

Objective: The various forms of mass spectrometry (MS) instrumentation have had a major impact on testing for analytes performed with clinical and forensic laboratories over the past decade. Improvements in MS instrumentation have led to the use of MS in many areas.

Methods: To highlight the value of MS testing, short reports are presented that are relevant to the following fields: pain management, transplant medicine, clinical toxicology, designer drug testing, genetic metabolic disorders, nutrition, dietary exposure to heavy metals, herbals and supplements, forensic pathology, pharmacogenomics, homeland security, performance enhancing drugs and peptides, clinical microbiology, physician licensing, and environmental exposures. These reports are based on real patients. The "stories" have been altered to comply with privacy regulations.

Results: Analysis of MS provides objective results that have an impact on many areas of medicine and society as a whole. Accurate analysis has an impact on guidance for medical practices.

Conclusion: The value of MS testing will continue to grow in the years to come.

Keywords: mass spectrometry, clinical toxicology, transplantation, pharmacogenomics, forensic medicine, heavy metals

Mass spectrometry (MS) has been an important research tool for academic research for many decades. In clinical practice, toxicologists have also relied on the combination of gas chromatography with mass spectrometry (GC-MS) for drug testing (qualitative analysis for confirmation of drug abuse and quantitative analysis for therapeutic drug monitoring). Developments within the last 20 years have enabled a more widespread implementation of MS into routine clinical laboratories. These include the automation of specimen extraction and injection, electrospray ionization enabling interface with liquid chromatographs (LC), matrix-assisted laser desorption ionization (MALDI) coupled with time-of-flight (TOF) mass analyzers for the analysis of large molecules, better data analysis tools, and improved reliability of instrumentation. Today, results from MS analysis, as conducted by licensed clinical laboratories, have reached an unprecedented scope. Nearly all branches of medicine have some routine diagnostic or research-based applications. Testing has also had an influence in solving problems in nonmedical issues. It is hoped that MS can be integrated directly into completely automated laboratory testing systems.

Abbreviations:

MS, mass spectrometry; GC-MS, gas chromatography with mass spectrometry; LC, liquid chromatograph; TOF, time-of-flight; ED, emergency department; NAPQI, N-acetyl-p-benzoquinoeimine; FDA, U.S. Food and Drug Administration; EPO, erythropoietin; hGH, human growth hormone; PEDs, performance-enhancing drugs; MALDI, matrix-assisted laser desorption ionization; ADH, alcohol dehydrogenase; BPA, bisphenol A; AGD, anogenital distance; CLIA, Clinical Laboratory Improvement Amendment.

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A particularly powerful tool is the use of high-resolution MS that enables molecular weight determinations to several positions after the molecular mass, which enables a determination of the molecular formula of an unknown compound. Using Internet searches instead of MS library matches greatly enhances the scope of the compounds that can be searched.

The purpose of this review is to present the scope of how MS testing influences patients' lives and those of their families. This goal is accomplished using a case report format,

whereby a patient or individual has an interaction with a medical system and testing is performed. These reports are based on real patients, who have been deidentified for adherence to privacy regulations. The individual stories have been embellished to make them more compelling. These reports illustrate the importance of MS in solving medical problems and producing objective information to make medical decisions. These stories also highlight how the inappropriate use or interpretation of MS analysis can lead to medical errors. They were written with a minimal amount of scientific details so that they could be understood by a lay audience.

Traditional Applications of MS

Pain Management

A female patient aged 27 years was seen for chronic abdominal pain and vomiting. She stated that she used acetaminophen chronically. In the emergency department (ED), her arterial pH was 7.12, her anion gap was 22 mmol/L, and her acetaminophen level was 3 mg/L. The most common causes of metabolic acidosis were ruled out because the lactate was normal and the ethanol, methanol, and ethylene glycol concentrations were below the detection limit. The ED requested a comprehensive urine drug screen to determine whether a more unusual cause of metabolic acidosis was present. Using a high-resolution time-of-flight mass spectrometer, a chromatographic peak was found with a molecular mass of 129.04. Based on this mass, it was determined that the molecular formula of this peak was $C_5H_7NO_3$.

A Google search for this chemical structure found a few candidate compounds: ethylisocyanatoacetate, 3-(hydroxyimino)-2,4-pentanedione, dimethadione, and 5-oxoproline, also known as pyroglutamic acid. While most of these compounds are organic solvents, a search for 5-oxoproline in the medical literature produced more of a biochemical hit. Pyroglutamic acid is one of the metabolites within the reduced glutathione degradation pathway that forms pyroglutamic acid and cysteine glycine.[1] It is well known that excess acute acetaminophen ingestion, such as seen in suicide attempts, depletes glutathione and leads to fulminant liver failure through the production of an alternate metabolite, N-acetyl-p-benzoquinoeimine (NAPQI), which binds to hepatocellular proteins to cause oxidative damage and mitochondrial dysfunction. Because NAPQI is highly reactive, it cannot directly be measured using MS. Instead, its presence can be detected by the adducts that it forms with cysteine, glutathione, and human proteins. Metabolic acidosis has been reported with chronic acetaminophen use, but it is a less uncommon complication and is not usually recognized.[2]

Transplant Medicine

Joan was a young adult during the 1960s and was part of the counterculture revolution occurring then. She attended large outdoor rock concerts including Woodstock. Joan also experimented with hallucinogenic drugs such as LSD and marijuana. Later, she began using intravenous drugs. While in her 30s, she contracted an unknown form of hepatitis ("non-A and non-B") from a contaminated syringe, which was later identified as hepatitis C virus. Joan eventually developed liver failure, and her only course of treatment was a liver transplant. Because of the shortage of cadaveric organs, she was put on the waiting list. Given her history of drug use, she was subjected to periodic urine drug screening. Joan had stopped using recreational drugs and passed all of the drug tests.

One day, Joan's general practice physician gave her trazodone for anxiety and chronic pain. A few weeks later, she turned out positive on the amphetamine drug screen and was temporarily removed from the transplant list. She denied using any recreational drugs and stated that she was taking a pain relief drug starting with the letter "t." The hepatologist assumed that the drug was Tylenol, and the specimen was therefore sent to a toxicology lab for targeted GC-MS confirmation. The result was negative for amphetamine, methamphetamine, Ecstasy, and acetaminophen. The hepatologist suspected that Joan was abusing a synthetic amine (bath salt) that triggered the positive immunoassay result. The specimen was sent for testing using a high-resolution LC-MS assay, which can find a wide spectrum of amines and metabolites. The result was positive for the presence of m-chlorophenylpiperazine, a metabolite of trazodone.[3] Joan's positive drug test result was overturned, she was exonerated, and she was put back on the transplant waiting list. But in the interim, and with the stress of these accusation, her liver disease worsened. Did she survive long enough to be treated with a transplant?[4]

Lab Medicine 2021;52;e58–e65 e59
DOI: 10.1093/labmed/lmaa083

Clinical Toxicology

A commercial pilot and his inexperienced copilot were landing their jumbo jet after a 12-hour international flight on a day when the visibility was fair. The plane flew too low as it approached the runway. The tail of the aircraft collided with the rocks below, ejecting some of the aft row seats and causing the deaths of the still-seat-belted passengers. The remainder of the plane crash-landed. When the aircraft came to a halt, its interior was on fire and the cabin was filling with smoke. Flight attendants immediately jumped up from their seats and opened the exit doors, which automatically inflated the evacuation chute attached to the door, and the remaining passengers began to exit the aircraft. However, another evacuation chute in the front of the plane malfunctioned and inflated inside the plane's corridor. In doing so, it knocked down and trapped 2 other flight attendants underneath it. When the pilots emerged from the cockpit, they saw their colleagues in peril. One pilot returned to the cockpit to retrieve the fire axe, and with the pointed end, he punctured the air bag and rescued the 2 flight attendants. They were unconscious and were sent to a hospital and admitted to an intensive care unit.

The 2 patients had been exposed to unreacted sodium azide, the chemical inside evacuation chutes and automobile airbags. In an emergency, the highly reactive azide is automatically ignited, which produces nitrogen gas and inflates the chutes. Clinical toxicologists consulted with the clinical laboratory to see if sodium azide can be measured in blood. Because the laboratory did not offer such testing, the toxicologists developed and validated a qualitative MS assay within 24 hours.[5] The results came out negative for azide. The flight attendants' prolonged hospitalization was not due to this toxic chemical.

Designer Drug Testing

Drew was an aspiring young actor who was in his apartment studying his lines for an upcoming audition. His agent, Jules, was reading the lines of the other characters in the script. Anxious and nervous, Drew made mistakes during the rehearsal. Jules decided that Drew needed to relax, so he went into the kitchen and decided to make some special brownies. Earlier in the day, Jules had purchased packets of a material labeled "Spice" from a local smoke shop. He knew that they contained synthetic marijuana. Jules opened 2 packets, mixed the drug into the brownie batter mix, and baked the dessert. An hour later, Drew was eating this treat. Drew became more relaxed and was able to rehearse his lines more effectively. Satisfied that he was making good progress, and with the time getting late, Jules went home. Shortly thereafter, Drew began to hallucinate under the influence of Spice. He became dizzy and disoriented. He called his spouse, who was working nearby, and told him that he was feeling funny. The husband left his job, went home, and immediately put Drew to bed. But later, when the spouse got up to use the bathroom, Drew arose, opened the window of their 4th-floor apartment, and jumped out. The fall caused major damage to his bones and internal organs, and he died a few days later from his injuries.

A toxicology analysis was conducted on Drew's blood and urine. Although the immunoassay screen was negative for cannabinoids, a high-resolution TOF-MS analysis revealed the presence of a compound identified as JWH-018, a synthetic cannabinoid of the naphthoylindole class. After a police investigation, Jules admitted to having laced the brownies with Spice. The district attorney found no purposeful intent to harm, and Jules accepted an involuntary manslaughter plea.[6] There are dozens of psychoactive synthetic cannabinoids that have been manufactured and released into the market. Untargeted LC-TOF-MS analysis is an ideal testing platform for detecting these drugs in the absence of standards for all of these synthetics.

Genetic Metabolic Disorders

A boy aged 3 months was admitted to the ED with vomiting, difficult breathing, and metabolic acidosis. His blood was tested using GC (without MS), which produced a peak with a retention time similar to that of ethylene glycol, an alcohol used as automobile antifreeze. The mother was suspected of poisoning, and the child was placed into child protective custody. She was granted a visit to her son. His illness relapsed a few days after the visit. Suspecting that the mother had poisoned her son again, police arrested her on assault charges. While the mother was in prison, the child died a few after his relapse, and the charge against her mother was upgraded to first-degree murder.. During her incarceration, the mother gave birth to a second infant boy. One month later, he developed symptoms similar to those of his deceased brother. With testing, the infant was diagnosed with methylmalonic acidemia, a genetic disease characterized by the production of propionic acid. In the meantime, the mother was on trial for murder. The infant's illness was not considered in the mother's defense. Ultimately, she was convicted and given a life sentence.

After the mother's story was featured on a television program, a chemist wondered if the original test on the deceased child had been misinterpreted given that MS analysis had not been used. The original specimen was retrieved and retested in independent laboratories.[7] This time, an LC-tandem MS assay was used. The assay found that priopionic acid and not ethylene glycol was present in the blood of the decedent. Armed with this new knowledge, officials reopened the mother's case; she was exonerated of all charges and was immediately released from custody. Laboratory errors can have significant consequences not only on medical care but also on forensic proceedings.

Dietary and Herbal Testing

Nutrition

Mei-Tse, a grandmother from Taiwan, was visiting her son and daughter-in-law in Northern California. One evening, she was babysitting her 7 year old grandson while his parents were out to dinner together celebrating their anniversary. Mei-Tse took the child out in a nearby field to pick fresh mushrooms. She found a mushroom that was identical in appearance to the nonpoisonous *Volvariella volvacea* species that Mei-Tse picked and ate near her Taipei home. Upon returning home, she cooked the toadstools with beef and noodles. Unbeknownst to her, this mushroom was poisonous. Shortly after serving the meal, she and her grandson became violently ill. She called her son, and the couple immediately left the restaurant. When they arrived home, an ambulance was there, and the paramedics were in the process of transporting Mei-Tse and their son to the ED.

An MS analysis of blood and urine confirmed the presence of several different amatoxin compounds from the *Amanita phalloides* mushroom species. Each of these compounds are arranged in a pentacyclic structure with proline- and tryptophan-derived residues and can be simultaneously extracted and tested using LC-MS/MS. A gasterenterologist told the parents about an experimental clinical treatment trial using milk thistle (silybum marianum) trial being conducted in Germany and received special permission from the U.S. Food and Drug Administration (FDA) to obtain the medication on an emergency basis to treat the patients.

When the package arrived with the milk thistle, the doctor realized that there had been a miscommunication because there was only enough medication to treat 1 of the 2 patients. The parents had to choose who would get the treatment first and who would have to wait until another shipment arrives. In the end, 1 patient survived and the other, who received a delayed dose, died.[8]

Dietary Exposure to Heavy Metals

Dylan came from a family of deep-sea fishermen. He had been interested in joining the family business from a very young age. His daily meals consisted of big game fish such as tuna, marlin, shark, and swordfish. His sister Diane did not like fishing and became a nurse at a local hospital. One day while attending a local seminar, Diane learned that people who ate a lot of seafood could be poisoned by mercury. Dylan's fingernails were dark, a sign of heavy metal exposure. Diane convinced her brother to see a doctor specializing in this field. A specimen of his urine was sent to a laboratory where an MS analysis using inductively coupled plasma as an ionization source was conducted. All of the heavy metals tested were within normal limits except for mercury. Dylan's doctor suggested that he undergo a "challenge" test in which he was given a chelator to drink before donating another timed urine specimen. Dylan was told that the chelator would release any mercury from his tissue stores and would give the doctor a more accurate assessment of Dylan's exposure to toxic dietary metals. Upon repeat testing, Dylan's urine mercury level was dramatically increased. Diane was told that chelation therapy would be necessary to clear the mercury from Dylan's blood, and she gave permission to proceed. A few weeks later, Dylan began experiencing muscle cramps, spasms, and tetany. Diane took him to the ED, where staff performed an ionized calcium test that revealed a critically low reading. An infusion of calcium gluconate is given, but it was too late, and Dylan died of acute hypocalcemia. Upon investigation of Dylan's death, it was determined that the wrong chelating agent was given and that he should not have been given any chelation in the first place, because his mercury levels were not increased. The chelation therapy given to Dylan had also reduced his calcium levels.

This case report highlights a potentially nefarious medical practice: A physician with ownership in a clinical laboratory recommends testing using a procedure that will likely produce a false positive result, in this case a heavy

Lab Medicine 2021;52;e58–e65 e61
DOI: 10.1093/labmed/lmaa083

metal measurement. The doctor then self-refers that individual to that doctor's own practice and convinces the individual that chelation therapy is needed.[9] The practice of heavy metal testing after a chelator challenge has not been condoned by American College of Medical Toxicology.[10]

Herbals and Supplements

Carl was a professional golfer who competed on the senior tour. When he was younger, he made a marginal living playing in one of the developmental tours. He had qualified for a few events on the main Professional Golf Association tour but was not good enough to stay there. Now that he was older than age 50 years, he believed that he was able to compete against the senior men. He entered and won a qualifying tournament on a Monday that earned him entry to the main senior event that weekend. Halfway across the world, Jimmy, a young Marines sergeant, was deployed in the Middle East. Jimmy was obsessed with physical fitness. In addition to the normal training and drills, he spent his spare time in the gym lifting weights. improve their performance. Carl ordered a natural erectile dysfunction herbal medication through the Internet called Trojan.[11] Jimmy purchased a supplement from the Marines base store called Oxy Jack, containing caffeine and another stimulant.[12] After Saturday's round on the senior tour, Carl was propositioned by an attractive younger woman for drinks and dinner. In anticipation of a continued encounter with the woman, Carl took a double dose of Trojan. In hopes of improving his workout intensity, Jimmy took a dose of Oxy Jack. At the height of their sexual and exercise activity, both men experienced chest pain and died of sudden-death myocardial infarction. Carl's female companion screamed at the sight of Carl's lifeless body and rushed out of the hotel room, and a fellow Marine found Jimmy dead near the bench press.

An autopsy was conducted on both individuals. Using LC-TOF-MS, hydroxyhomosidenafil, an analog of the active ingredient found in Viagra, was found in Carl's postmortem blood. Dimethylamylamine was found in Jimmy's blood. Standards were not available for either analogs. These reports highlight the dangers of herbal medications. Because the FDA considers these compounds to be nutraceuticals, there is little oversight regarding the manufacturing, sale, and distribution of these potentially dangerous substances. When the cause of death was released, the Marines base stopped selling Oxy Jack in its stores.

Forensic Pathology

Max was an elderly man who lived in a nursing home. He was regularly visited by his daughter, Gail, every Sunday evening. On her last visit, Max seemed tired and sleepy but was otherwise conversational with his daughter. She thought that nothing was unusual and left. The next day, Max was found dead in his room. An autopsy revealed high concentrations of quetiapine in his blood. The coroner ruled the death as natural. When questioned about the high quetiapine level, the coroner concluded that it resulted from postmortem redistribution. As part of the putrefaction process after death, drugs leach out of organs and tissues into blood pools, thereby increasing the concentration relative to premortem values. The extent to which this process occurs depends on a number of factors including the lipid solubility of the drugs, the temperature of the cadaver, and the duration of time from death to autopsy.

Gail challenged the coroner's conclusions. Under a court order, she obtained her father's pharmacy and nursing records, which confirmed the presence of quetiapine and prochlorperazine used as anti-depressants and to treat his anxiety. Gail became suspicious when the home told her that the nursing records from the night before Max's death were missing. She contacted a toxicologist to review her father's records and the postmortem blood MS analysis data from the autopsy record. The toxicologist confirmed that the quetiapine level was in the toxic range but noticed that the prochlorperazine level was within the therapeutic range. Because both drugs were given regularly and have similar pharmacokinetics, he questioned why 1 drug redistributed after death and the other did not. Armed with this information, Gail brought a lawsuit against the nursing home. The night nurse was questioned about the missing nursing notes. Under oath and duress, she admitted that it was customary to give Max an extra dose of quetiapine on some nights when he was agitated and "unruly." The drug would calm Max, enabling him and the other residents to sleep. Gail received a satisfactory settlement offer from the nursing home's attorneys.[13]

Nontraditional Applications of MS

Pharmacogenomics

Nick was a pharmaceutical sales representative who self-medicated with Tylenol 2, a combination of codeine and acetaminophen. He occasionally used this drug for pain relief, having suffered an automobile accident a few months earlier. As part of his daily routine, Nick frequently traveled by car, visiting physicians and other clients. He was returning home after a long day on the road and accidently hit an elderly pedestrian. The woman was fatally injured. Nick was tested for driving while under the influence of drugs and alcohol. His breath test was negative for ethanol, but his urine was positive for opiates. Nick was tried for involuntary manslaughter. The presence of opioid narcotics was sufficient for a conviction, and he was sentenced to 10 years in prison.

A few months later, Nick was reviewing the evidence used in his conviction. The LC-MS confirmation analysis showed a high concentration of codeine but only a trace amount of morphine, the major active metabolite of morphine, and a high concentration of morphine-3-glucuronide. As a former pharmacist, Nick knew that to obtain full analgesic activity, codeine as a prodrug must be converted to morphine. He wondered if he might be one of the few individuals who are genetically poor metabolizers. He contacted his lawyer, and they requested a court order to have his blood collected and his genes tested for cytochrome P450 and UGT-glucuronsyl transferase variances. These genes encode for enzymes that participate in the metabolism of codeine. The results showed that Nick was homozygous for the CYP*4 variant, a gene that produces no enzyme activity, and heterozygous for UGT 2B7*2, a genotype associated with increased glucuronidation. Armed with the new evidence, Nick's attorney petitioned the court to reopen his case. Nick and his attorney showed because of his genetic makeup, he was unable to produce morphine to any appreciable extent and that what little morphine he could produce was metabolized into the inactive morphine glucuronide. Nick's conviction was overturned, and he was released from prison.[14]

Homeland Security worked in his father's rug factory. He was an avid soccer player and on weekends played on various teams in the city. During 1 game when it was particularly warm, Frank collapsed on the field. An ambulance was called, and he was taken to the ED. A co-oximeter result showed a low oxygen saturation and a high methemoglobin concentration. Frank was treated with methylene blue and over the next few hours, his oxygen status improved. To investigate the cause of his oxygen desaturation, his blood was sent for glucose-6-phosphate dehydrogenase testing, which came out normal. Serum and urine specimens were sent to the toxicology laboratory to determine whether he was exposed to oxidizing chemicals. Using high-resolution TOF/MS, the laboratory reported the presence of nitroaniline. This chemical is used as an intermediate in the synthesis of dyes. Because Frank worked in a rug factory, investigators from the poison center were sent there to determine if other workers could be exposed to this chemical. They found that the company was not a manufacturer of carpets but was simply an importer; as such, there were no chemicals present. Further investigations by the toxicologists learned that nitroaniline is a metabolite of tri-nitrotoluene, better known as dynamite. Investigators obtained a court order and went to his home, where they found bomb-making equipment. Frank was a terrorist, and a plot was averted.[15]

Performance-Enhancing Drugs and Peptides

Lenny was the son of the head of the transfusion medicine lab at major medical center. As a teenager, he used to spend summers working in his father's blood bank as a research assistant. While he was working there, he learned a lot about blood doping practices. This knowledge became extremely useful when Lenny took an interest in competitive bicycle racing. Although he was tall, lean, and a strong rider, his times were not good enough to win local races. Without his father's knowledge, Lenny went into the blood bank at night and removed units of his own blood, secretly stored it, and transfused it just before races. This procedure improved his endurance and stamina to the point where he became one of the best riders in the region. Over the ensuing years, Lenny set his sights on qualifying for national cycling teams, with the hopes of competing in the Olympic Games. In addition to his autologous transfusion abuse, Lenny began using recombinant erythropoietin (EPO) and then human growth hormone (hGH) to further improve his performance.

In the meantime, Olympic drug testing labs made significant analytical testing advances to detect performance enhancing drugs (PEDs) and hormones. Recombinant EPO use can be determined by isoelectric focusing and double immunoblotting. Differences in glycosylations between

DOI: 10.1093/labmed/lmaa083

these 2 forms may also be useful as measured by MS analysis. The protein is digested with trypsin to produce specific measurable peptides. The main hGH isoform is a 22 kDa peptide.[16] There is also a 20 kDa peptide that is produced by alternate splicing. The typical ratio of the 22:20 kDa hGH peptides is 12:1.

After Lenny qualified for the US national cycling team, his blood was tested for doping. They found an increase in the ratio of the hGH peptides, and Lenny was disqualified from the competition. He stopped using PEDs, but his race times were no longer competitive.

Clinical Microbiology

Terry and Tommy were identical twins. When they graduated high school, they decided to go to separate US colleges. Terry attended a West Coast school, and Tommy enrolled in a college on the East Coast. During the Thanksgiving holiday break, the boys returned home. After attending a party with friends, they contracted a bacterial infection after eating the same spoiled food. The symptoms were originally mild, and the boys returned to their respective schools. But their condition worsened, so they each sought medical attention at their university hospitals. The clinical laboratory staff at Tommy's university cultured his blood. Failing to grow anything for 2 days, the doctor ordered a wide-spectrum antibiotic. For Terry, a molecular test produced a positive result for *Escherichia coli* within a few hours after blood collection. Furthermore, using MALDI-TOF MS, the clinical laboratory also revealed that Terry's infection was resistant to carbapenem, a beta-lactam class of antibiotics. Based on these data, dual therapy consisting of colistin and polymyxin B was given. Despite the best efforts, Tommy died and Terry survived. He did not know that his twin brother was also sick because there was poor communication between the doctors treating them at each school. In this situation, differences in the clinical laboratory technology used resulted in a significant disparity in the outcomes of 2 individuals who had identical genetics, infection, and route of infection.[17]

Physician Licensing

Dr. Schaefer was a gynecologist in private practice who was depressed after the death of a patient and began drinking alcohol. One day at the office, her colleagues noticed that she was slightly off-balance. The head of the group confronted Dr. Schaefer with the suspicion that she might be under the influence of alcohol. She was taken off the care of her patients and asked to undergo a breath alcohol test. When a blood alcohol level of 0.05% was produced, Dr. Schaefer was reported to the state's physician licensing board, and her license was suspended. In her defense, she claimed that she drank 3 glasses of wine the night before and should not have had alcohol in her system that morning.

After researching the literature, Dr. Schaefer learned that some individuals are slow alcohol metabolizers (possessing gene variants of alcohol dehydrogenase [ADH]). Her attorney petitioned the state to conduct a clinical study to prove that Dr. Schaefer was such a person. The lawyer contracted with a local laboratory to have 5 hourly blood specimens taken after drinking 3 glasses of wine with a witness. The laboratory measured her alcohol levels and found that instead of the usual 15 mg/dL/h metabolism rate, hers was 3 mg/dL/h. Dr. Schaefer and her attorney convinced the licensing board that she did not know that alcohol was still in her system after 12 hours, and she vowed to not drink on the night before work again. The board reinstated Dr. Schaefer's license.

A toxicologist at the lab learned of this analysis and suspected that the doctor may have taken fomepizole, a drug that retards ADH, before drinking. Using a targeted MS analysis, he found the drug present and was able to prove that the doctor was deceiving her practice. However, Dr. Schaefer's attorney prohibited the release of these data due to attorney-client privileges. Months later, Dr. Schaefer, while impaired, performed a minor obstetrical procedure on a patient.[18]

Environmental Exposures

Roma worked in a tollbooth. If drivers wanted a receipt for their payment, Roma handed them one that was printed on thermoprinter paper. These printers use a thermoactive dye called bisphenol A (BPA). When Roma became pregnant, she continued to work and unknowingly exposed herself and the fetus to BPA. At the time, BPA was also found in many other household items including canned goods, water bottles, other plastic containers, and hospital tubing. Research has shown that BPA is one of several compounds that are classified as endocrine disruptors, meaning that they alter the normal hormonal balance between masculinity and femininity. This disruption affects developing fetuses in utero more so than in adults. In addition, BPA and phthalates used to

make plastic soft and pliable are estrogenic, so exposure causes a feminization of the patient.

The in utero effects can also alter the anatomy of the growing fetus. One measure is the anogenital distance (AGD), which is the distance between the anus and the genitalia (scrotum or vagina). Males exposed to BPA and other estrogenic disruptors have a shortened AGD. These individuals have lower semen volume and sperm count and a higher likelihood of subfertility or infertility.

Laboratory technicians can use LC-MS to measure BPA in the urine of exposed individuals. However, this procedure requires the removal of all potential sources of contamination, including the tubing used with the MS analyzer itself. In addition, the analysis of BPA and phthalates require the use of MS analyzers that have extremely high analytical sensitivity.[19]

Roma delivered a baby boy who exhibited hypospadias (the opening of the boy's urethra was not at the tip of his penis). It was surgically repaired when the child reached the age of 9 months. The pediatrician expected that the boy would suffer from other developmental problems. Because of concern about abnormal child development, BPA has been banned by the FDA for use in infant formula packaging, baby bottles, and sippy cups. The chemical is still used in other food containers.

Conclusions

Under the Clinical Laboratory Improvement Amendment (CLIA), MS analysis is considered to be of high complexity. Because there is an absence of FDA-cleared MS assays, laboratories wishing to report clinical results must validate assays under the Laboratory Developed Test provision of CLIA. Technicians performing MS analysis undergo additional training to operate and maintain the instruments. Untargeted MS data analysis for toxicology unknowns requires additional training and experience. As shown in these case reports, analysis of unknowns can be very useful in solving medical mysteries. This potential could be helpful in justifying to administrators the need for equipment and personnel to operate MS equipment.

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DOI: 10.1093/labmed/lmaa083

Special Report

Pathology—The Beginnings of Laboratory Medicine

First in a Series

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Laboratory Medicine 2021;52:e66-e82

DOI: 10.1093/labmed/lmaa098

How does it make laboratory professionals feel when no one really knows what we do? Our career—the profession we chose for a living—where we spend most of our time? When all that we do for patient care, and our accomplishments every day, go unnoticed by the general public? How unsettling is it when very few grasp and understand the importance and significance of who we are? As practitioners in laboratory medicine, how often have we heard these questions?

"You seem so smart. Why did you not become a doctor?"
"You are important in healthcare. Are you a nurse?"
"So—what do you do in pathology and laboratory medicine?"

Do we really know how to respond? "I work in the laboratory" is not at all a sufficient response. It does not take into consideration the education, technical skills, body of knowledge, scope of practice, or independent judgment required of laboratory professionals.

When we consider the fact that laboratory testing significantly and critically 'aids in the detection, diagnosis and treatment of disease' - a more respectful response is worth pursuing. To help ourselves and in turn help the general public (and maybe some colleagues) whom we serve to understand and appreciate pathology and laboratory medicine, we need to understand what pathology and laboratory medicine are and the people behind it.

Abbreviations:
RBC, red blood cell; WBC, white blood cell.

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What Is Pathology?

In essence, pathology is the study of disease with 2 main divisions: anatomic pathology (the study of body parts) and clinical pathology (the study of body fluids). The earliest application of pathology began and was rooted within the development of medicine—most early pathologists were also practicing physicians. Considering that today the laboratory professions are an underappreciated field, it is important to follow the history to the current day. This article sheds some light on how laboratory medicine evolved and observes that the history of pathology is complicated and convoluted.

Historical Timeline of Laboratory Medicine: Early Laboratory Techniques

Laboratory medicine began with the study of a natural byproduct of all humans that was easily obtained: urine. Urine specimens today can provide valuable information for a physician. Even if many current laboratory professionals hold opinions of urinalysis as perhaps not the most popular

area assigned for work, urine testing in itself has been quite diverting and dramatic from the very beginning. Thousands of years ago, early providers relied on much different, primitive methods of analysis and depended heavily on assumptions based only on observations.

4000 BC

Based on unearthed papyrus, Babylonian and Sumerian "physicians" were recording urine evaluations on clay tablets. Early Egyptian medical assessments were also recorded on scrolls and were considered to be advanced. However, the luxury of attaining a diagnosis of disease was originally predominantly allocated solely to members of the royalty and the wealthy. Rituals and sacrifices (ie, gods/demons) were generally used otherwise to determine the nature of disease. In either case, patient care was very much left to chance.

300–100 BC

Ancient Hindu physicians conducted testing by pouring urine on the ground to observe whether ants would be attracted to the sweetness of urine.

Classical Era

400–301 BC

The Greek physician Hippocrates (460–370 BC), known as the "Father of Medicine," had an indelible impact on Greek culture during the Hellenistic era that was exported directly to Roman medicine. Hippocrates was one of the first physicians to interpret body functioning based on urine. Professing that no other organ system of the human body provided so much information by its excretion as does the urinary system, he determined that urine could be used as a prognostic indicator for the examination of illness. He utilized human senses such as eyes and ears. For example, seeing sediment correlated with increased fever, and seeing bubbles floating indicated that the disease would be prolonged. Hippocrates also professed the importance of smell and even taste. His hypothesis followed that urine was a filtrate of 4 body fluids (humors), each originating from a different region of the body. Each humor needed to maintain balance for health.

One of the first manual laboratory tests ever performed: the tasting of urine (confirming its sweetness).

101–200 AD

Approximately 6 centuries later, Aelius (Claudius) Glenus (129–216 AD)—popularly known by the name Galen—was a Roman Empire physician born into a wealthy Greek family—who expanded on the ideals of Hippocrates. Considered as perhaps the greatest medical figure of that time, he clarified that urine was not a filtrate of the 4 humors (blood, phlegm, yellow bile, black bile) and proposed that urine was a filtrate of only the blood. He also suggested that liquid intake by a healthy patient was proportional to output. Galen's writings were extensive, and his theories on anatomy (via living animals, because human dissections were prohibited by Roman law) directed medicine up until and throughout the Middle Ages. The undisputed acceptance of his views (ie, blood is created in the liver after ingesting food and flows to either the right or the left side of the heart) dominated medical thinking for approximately 1,500 years, which unfortunately did not promote new investigations with new ideas to challenge—and prolonged unproductive thinking.

Early Middle (Medieval) Ages (500–1300 AD)

Medicine during these years was influenced mainly by Arab and Byzantine physicians (mostly monks) and held to the basic theory that disease was an inevitable outcome of deemed punishment—symbolic of sinning and/or witchcraft.

The 7th-century Byzantium physician Theophilus Protospatharius (610–641) authored the first manuscript published solely on urine. Held in high esteem, "De Urinis" reviewed the subject according to the ancient classical conditions attributed to urine but concentrated on its characteristics to define disease, migrating from a theory of disease to clinical application. The study of urine remained the main source of laboratory testing for centuries, as did recognizing that the combination of clinical symptoms with standardized laboratory measurements could determine clinical disease. One of the first actual laboratory techniques ever performed was 500 years after Galen: precipitating protein with heat, causing urine cloudiness. Urine had become the primary diagnostic tool for laboratory testing.

The 10th-century Egyptian/Jewish practitioner Isaac (Ben Solomon) Judaeus Israeli (850–950) was the founder of the origins of nephrology. He widened both the writings of Protospatharius and the ideas of Galen and was an enormous contributor to Arab-Jewish medicine— history

Lab Medicine 2021;52;e66–e82 e67
DOI: 10.1093/labmed/lmaa098

records him to have lived 100 years. Israeli also originated the concept that fluid and sediment from blood seeped into the kidney and from the kidney to the bladder. The analysis of urine became a complex procedure used for both prognostic and diagnostic purposes with disease. Under the Jerusalem Code of 1090, urine became the exclusive diagnostic tool of legal necessity. If a physician failed to examine a patient's urine, then public beating or fining were the punishment outcomes.

The 12th-century royal Islamic Persian physician Zan al-Din Sayyed Isma'il ibn Husayn Gorgani (al-Jurjani; 1040–1136) required his patients to collect 24 hours of urine into a clean vessel on an empty stomach after a good night's rest (this was not inordinately difficult or burdensome, despite the fact that laboratory staff were not available to advise on the preanalytical variables of proper collection and storage of specimens).

The 13th-century Byzantine physician Johannes Zacharias Actuarius (1275–1328) authored a comprehensive 7-volume manuscript on just urine. This manuscript was the most complete work on this subject matter and remained so until the chemical developments of the 19th century. He detailed how to collect 24-hour urine: using a clean, transparent, very large bladder-shaped bottle protected against heat, cold, and sunlight.

This medical practice of urine testing (visually examining urine) was collectively known as uroscopy ("water casting") and eventually became associated with the Urine Wheel, a diagram linking the color of urine to disease. The variations of urine smells and tastes were also displayed. The medieval physician held up a glass flask (a matula) of the patient's urine and would compare it to the colors on the wheel. The urine flask mimicked the shape of the bladder to preserve the urine in its familiar environment and became the symbol of the physician in medieval medicine (**Figure 1**). The Urine Wheel became well established and was an immensely popular quick reference chart for 20 different types of urine. Almost every urinary characteristic of color, smell, and taste was directly related to the disease state of the patient. Of course, the urine had to be examined immediately to maintain the warmth from urination, and various lighting had to be considered. What is most perplexing is how urine remained such a viable body fluid throughout the Middle Ages.

An entertaining sidebar is that as an instructional aid for students in Salernes, France, a poem was written by French royal physician Gilles de Corbeil (1140–1224) to sing along with to help with memorizing the 20 types of urine and became a classic.

Most of the testing performed in medieval days by today's standards would be considered a fad or mockery (eventually referenced satirically in Shakespearean plays during the 1600s). Yet what transformed into urinalysis today still withstands the validity of direct observation, ie the color of urine (red, indicating the presence of red blood cells [RBCs]), and brown (indicating the presence of bilirubin) and a foul odor (indicating infection). Fortunately, tasting is no longer required, although collection still involves urinating into a cup in place of a flask. How far have we really come?

13th century (1201–1300)

These early "laboratory tests" developed were performed by physicians, who were predominantly men, until Modino de' Luzzi (1270–1326) at the University of Bologna employed Alessandra Giliani as a medical/surgical assistant to perform laboratory testing such as surgical dissections. Using the cadavers of criminals, he "supervised" autopsies and incorporated them into medical training, gaining the title "Restorer of Anatomy." Meanwhile, as those times would have it, a young woman involved in medical testing was not considered acceptable, and historians are hard pressed to find definitive evidence of Giliani's work, which is assumed to be either lost or destroyed. Distinguished as the first female anatomist, she ironically died of a septic wound—a laboratory-acquired infection—at the very young age of 19.

Late Middle Ages to The Renaissance (1301–1600) AD —A Time of Rebirth

After the Crusades (1095–1492), the monasteries were where ancient medicine continued under monks who became physicians, and this practice did not alter significantly until the 14th century and the revival of interest in Bologna universities. Although this century was an impressive period with advanced artistic, cultural, literary, and philosophical revelations and political and economic growth, there is no mention of pathology and laboratory medicine in historical records, and for good reason. Those advances were not yet forthcoming. Learning was primarily for the sake of knowledge, and none existed in application of the laboratory.

Figure 1

Medieval Uroscopy

There were some advances in pathology noted—but again, predominantly through academia—with a renewed interest in ancient Greek and Roman medicine along with Arabic/Persian medicine. However, some key medical discoveries did pave the road for modern medicine.

Swiss/German physician/alchemist Theophrastus von Hohenheim, or Paracelsus (1493–1541), referred to the balance of minerals and chemical remedies in medicine (pre-chemistry/pre-pharmaceutical) and is sometimes called the "Father of Toxicology." He advocated fasting instead of bloodletting/purges and anticipated early germ theory: illnesses were the outcome of outside agents attacking the human body. He discovered that using vinegar (acid) resulted in cloudiness in urine hundreds of years after Protospatharius used heat (early studies of proteinuria).

Leonardo da Vinci's (1452–1519) contributions are lesser known in the medical field. This multitalented renaissance man never ventured into the enticing world of laboratory medicine, but he did recognize the need for scientific knowledge and afforded anatomical techniques and detailed human anatomy sketches (anatomic art), which greatly enhanced knowledge of the human body.

Although today the dissection of bodies, organs, tissues to study disease is a vital component of pathology laboratory medicine, slow progress was made over the centuries because of the Hippocratic and Galenic influences and the periodic prohibition of dissections. An early Arabian physician known by one name (Avenzoar), Abu Marwan Abd al Malik Ibn Zhr (1091–1161) reportedly made the first postmortem dissections.

The 15th-century Florentine physician and skilled diagnostician Antonio di Paolo Benivieni (1443–1502) published "The Hidden Causes of Disease," which was one of the first works proposing anatomic pathology as a separate

Lab Medicine 2021;52;e66–e82
DOI: 10.1093/labmed/lmaa098

specialty. His skilled autopsy techniques set him apart as the "Founder of Pathology."

The 16th-century French surgeon Ambroise Pare (1510–1590), who moonlighted as a barber, served 4 kings and became noted as the "Father of Surgery and Forensic Pathology," inventing several surgical instruments and techniques.

The greatest advancement in anatomic pathology finally came with Bologna's Giovanni Batista Morgagni (1682–1771), who became known as the "Father of Pathologic Anatomy." As the most famous of the early gross pathologists, he raised standards and improved existing knowledge in every area of pathology. The mostly clandestine postmortem examinations of the past centuries now became open-to-the-public theaters. Morgagni directly recorded and correlated the underlying diseased anatomy of organs with clinical symptoms and illnesses in over 640 autopsies; this labor is considered the beginning of modern medicine and pathology. However, pathology was not yet dignified as a separate science and so remained part of clinical medicine.

17th Century (1601–1700)

Many practitioners continued using ancient and superstitious rituals and cures during the 17th century. The use of "medical equipment" for bloodletting and purging became acceptable practice, which turned patients away from medicine. Physicians could render a diagnosis without ever seeing a patient in person by just examining the urine. Uroscopy became available to the common person at home, including self-diagnosis. Amateur laymen became the new healers. Chlorosis (referring to lovesickness) and chastity were also diagnosed long before the immunoassay measurement of glycoprotein hormones such as human chorionic gonadotropin. "Uromancy" or "urimancy" followed as the analysis of one's urine for fortune-telling and witch-hunting. A common practice was to place iron nails or needles or pins in a bottle of urine with a secured cork. It was taken as a sign of guilt if the suspect (or patient) became ill or if the cork popped out.

Of satirical interest today would be the very popular book "The Pisse-Prophet," written by Colchester (England) physician Thomas Brian in 1637 decrying the contemporary medical practice of uroscopy. He advised practitioners to simply place the urine flask on a windowsill to allow for sedimentation while asking the courier who transported the flask questions about the sick patient. Basically, without any scientific or real clinical testing, he advised practitioners to exaggerate the extent of disease. If patient lived - skilled practitioner; but if patient died - then claim the correct prediction had been made. Laboratory testing definitely needed to be developed.

The most notable advancement perhaps was English physician Thomas Willis (1621–1675), who coined the term "mellitus" (diabetes), basically to describe what had been perceived for centuries—that certain patients' urine had a different taste.

Uroscopy eventually lost its solo appeal by the end of the 17th century, opening the door for another major body fluid to become the main focus of study and leading to more noteworthy and pertinent laboratory testing: blood.

Practitioners of ancient medicine realized that blood was a notable "fluid of life": phlebotomy (bloodletting) was practiced, Hebrews drained animals' blood before consuming them, and Romans drank the blood of their enemies. There were, however, no specific scientific laboratory blood tests throughout the centuries until the microscope was created to examine blood beyond the naked eye (detailed in the following section).

Of interest are some impressive digressions from past accepted theories, finally moving forward toward modern laboratory medicine. For example, Syrian Muslim anatomist Ibn al-Nafis (1210–1288) is believed by many historians to be the first to challenge Galen's findings by proposing the existence of pulmonary circulation. How al-Nafis arrived at this hypothesis has been controversial because dissections were prohibited by Islamic law. Unfortunately, his medical encyclopedia manuscripts were incomplete and unpublished and many volumes were lost.

Spanish physician Michael Servetus (1511–1553) was the first European who incidentally referred to pulmonary circulation but within theological writings and unorthodox teachings of religion. For this he was executed (burned alive) in 1553.

The extraordinary breakthrough with pulmonary circulation is often credited to English scientist William Harvey (1578–1657). He refuted the allegations of witchcraft and purging and bleeding. In 1628, Harvey revolutionized the concept

of disease in his groundbreaking detailed description of systemic circulation—the property of blood being pumped from the heart to the organs of the body (although he erred by not noting the capillaries). Harvey's published writings finally disputed Galen's long-established accepted theory of separate arteries (vital) and venous (natural) systems. Despite facing much skepticism during the 17th century (his practice encountered much criticism), he chose a diverged road for others to travel—a paved road that was enhanced with the development of the microscope.

The Invention and Use of the Microscope

The microscope was actually what intrigued many future laboratory professionals—the author included—about a career in the laboratory and the world beneath the microscope. Although the microscope was somewhat limited in use during the 17th and 18th centuries, the creation of optically corrected lenses and objectives augmented microscopy. Although lenses were used for eyeglasses dating as far back as the 13th century, they now had a higher purpose. From the mid-19th century and thereafter, what occurred in vivo could actually be appropriately studied in vitro: no more guessing, no more assuming, and much less speculating. The ancient premise relating disease found in from nature to sin or worse, witchcraft, was finally replaced with scientific measurements and anatomic autopsies, which viewed the body in both harmony and disease using localized processes.

A level of controversy exists as to exactly who invented what and when. Galileo Galilei (1564–1642) was an Italian astronomer who has been associated with the invention of the telescope, which led the way to the invention of the microscope. However, many historians credit the first telescope to German-Dutch glassmaker Hans Lippershey (1570–1619), who in 1608 actually acquired the first patent. Lippershey is also alternatively suspected to be the inventor of the first microscope in 1590.

Meanwhile, father-and-son team Hans Janssen (1605–1633) and Zacharias Janssen (1580–1630) were Dutch spectacle lens makers who later designed devices used to magnify objects. Zacharias (still a teenager) has more often been credited with creating the first so-called compound microscope (more than 1 lens) by placing a lens on the top and bottom of a tube and noting that objects on the other side were suddenly magnified in size, although the images were blurry and the device had no resolution capabilities.

The first to actually utilize the microscope for laboratory testing was Jesuit German priest Athanasius Kircher (1602–1680), declared the "last Renaissance Man." As early as 1646, he examined the blood of plague victims (one of the first to do so). In 1658, he recorded the origin of disease via "worms" in the blood—although note that a low-powered microscope could not possibly discern the *Yersinia pestis* bacteria at that time, so he may have actually observed increased numbers of white blood cells (WBCs). Of particular interest, Kircher also proposed hygienic measures to prevent the spread of disease, such as isolation, quarantine, and wearing face masks to prevent the inhalation of "germs."

Dutch scientist Jan Swammerdam (1637–1680) is respected as one of the most accurate of classical microscopists, having cultivated new techniques for examining/preserving/dissecting specimens specifically to view blood vessels. In 1658, he was the first to observe RBCs under the microscope by researching insects. He never practiced human medicine despite completing his medical studies.

Marcello Malpighi (1628–1694) studied animals and for the first time in 1661 observed "very small red particles" as blood composition. Referred to as the "Founder of Microscopical Anatomy/Histology," this Italian physician discovered the link between arteries and veins (capillaries) and discovered a human blood clot in 1666.

English scientist Robert Hooke (1635–1703) made contributions in many fields of science, including refining the compound microscope and introducing the iris diaphragm (**Figure 2**). Using a microscope in 1665, he coined the first use of the word "cell" (reminiscent of the small rooms where monks lived) while observing microscopic cavities in cork. His research also focused on microorganisms as the etiological agent of infectious disease.

By 1830, the English amateur opticist and physicist Joseph Jackson (JJ) Lister (1786–1869) had designed a lens combination reducing achromatic aberration (where objects appear colored) and spherical aberration (where objects appear circular), improving image resolution for minute detailed identification. This development solidified the microscope as a superior performance piece of laboratory equipment. Lister did all this during his spare time from his main occupation in the wine business.

Lab Medicine 2021;52;e66–e82 e71
DOI: 10.1093/labmed/lmaa098

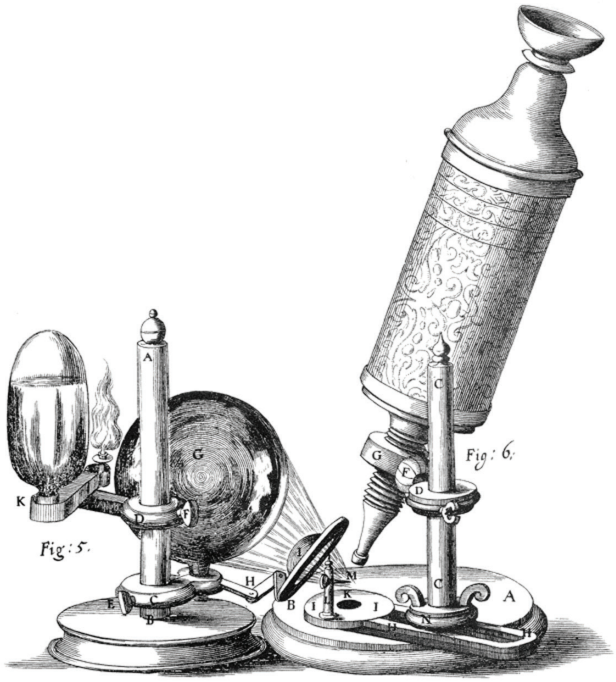

Figure 2

17th century microscope

Over the years, with advancements made to the optical abilities of the microscope, cellular and bacterial knowledge along with laboratory testing broadened tremendously and pathology found its origins within clinical microscopy, but it was still a long way from the advanced superior fluorescent and electron microscopes of today.

Beginnings of the Clinical Laboratory

18th century (1701–1800)

During the 18th century, internal medicine practitioners developed new drugs and precision equipment for the doctor. Textbooks of pathology diagnostic testing were being published, but much information still needed to be assimilated into clinical laboratory testing. With the studies of illnesses now centered around the microscope as the key piece of laboratory equipment, the concept of diseases soon developed from organ-based to cell-based and cellular components.

Microbiology

There was ultimately a correlation of symptoms with underlying disease: the birth of the new "pathology". Microbiology is probably the oldest known of all laboratory areas with tapeworm infections being recognized as one of the earliest parasites - discovered by Egyptians from 1500 BC.

However, Dutch textile merchant, businessman, and self-taught microscopist Antonie van Leeuwenhoek (1632–1723) is generally regarded as the "Father of Microbiology". He pioneered microscopy with his hobby of grinding lenses to study objects. Ironically, in 1676, unsatisfied with the magnifying lens available, he designed his own lens to examine the quality of thread in his draper shop. Much to his amazement (and that of the scientific community), he was the first to "accidentally" observe "animalcules" (microbes, or unicellular organisms) in water. These were among the first bacteria discovered, laying the foundation for the widespread detection of bacteria, which were not fully understood until 200 years later. Leeuwenhoek further observed sperm in fish, mammals, and humans. His unique lens design

techniques were not reproduced until the late 1950s; this accomplishment was not bad for a businessman with no medical background.

Hematology

British physiologist William Hewson (1739–1774) is often referred to and distinguished as the "Father of Hematology" for his many considerable discoveries in all hematological areas of study. He described the RBC, one of the most intriguing of the body's cells, as discoid, refuting the spherical shape originally described by Leeuwenhoek, who had detailed observations and illustrations of "red particles" in blood in 1695. Hewson incorrectly supposed that RBCs started as the rare "colorless cells" (WBCs) and that the center of the RBC was a nucleus. It is important to note that at this time, lenses were nowhere near perfected. Furthermore, Hewson's belief in the presence of cell membranes was largely ignored. However, his isolation of the protein fibrin and his discoveries that fibrinogen (not cells) leads to the coagulation process and that lymph vessels exist (at least in animals) greatly advanced knowledge about the lymphatic system. Hewson died of sepsis infection at age 35 after cutting himself.

Chemistry

Originally referred to as "alchemy" (defined as the transmutation of elements; practitioners were primarily attempting to convert metals to gold) and based on mystical beliefs and not the scientific method, chemistry began initially as a qualitative testing of chemicals and minerals. Some documents refer to the first chemical test as the one used to determine pregnancy. Egyptian women in 1350 BC urinated on seeds of wheat and were deemed pregnant if the seeds sprouted.

French chemist Antoine-Laurent de Lavoisier (1743–1794) was, according to historians, a pivotal influence on the chemical revolution. He suggested the law of the conservation of mass (a substance may change in form but retains the same mass); this idea began the conversion in science from qualitative to quantitative analysis. Identifying oxygen and hydrogen, de Lavoisier laid the foundation for the fundamentals of chemical reactions and is called the "Father of Modern Chemistry." He constructed the metric system, wrote the first list of elements, and reformed chemical nomenclature. Unfortunately, because he had political adversaries because of his economic funding for scientific research, he was charged and sent to the guillotine, becoming a victim of the French Revolution.

Because of the limited analytical development of equipment, only simple chemical tests were available until the early 19th century. These simple tests, such as the examination of stones and urine, could be performed at the bedside. One might say that they were the first point-of-care tests.

Immunology

English country doctor Edward Jenner (1749–1823) is attributed with the discovery of the immune system and is hence referred to as the "Father of Immunology." Coincidentally, because of his familiarity with farm work (in particular, dairy cows), in 1796 he inoculated a 13-year-old boy with the first vaccine for cowpox, which made the boy immune to smallpox.

Histology

French pathologist Marie Francois Xavier Bichat (1771–1802) was an army surgeon during the French Revolution who used freshly guillotined bodies to investigate tissues. By 1801—and supposedly without the use of the fundamental microscope—he identified 21 types of tissues. He redirected the study of pathology from focusing on organs to the concept that disease occurred as the result of pathological alterations in tissue. Long after his untimely work-related death, he came to be known as the "Father of Histology." The German word *Histologie* was coined almost 20 years later in an 1819 book by anatomist August Franz Josef Karl Mayer (1787–1865).

Although histological progression moved slowly and it was not until the 19th century that histology became an independent discipline, the following tools and practices utilized in histology remain critical pieces of equipment.

Microtome

Czech physiologist Jan Evangelista Purkinje (1787–1869) was one of the most highly regarded scientists of his time. This Bohemian professor was the founder of laboratory training in connection with university teaching in Germany. Although microscopes with advanced magnifications and resolutions were in use by the 18th century, the process of tissue cuttings lagged far behind, producing only thick sections. Manual preparation using razor blades and knives

Lab Medicine 2021;52;e66–e82 e73
DOI: 10.1093/labmed/lmaa098

evolved by the 1770s into the use of hand-operated "compressing" devices devised to create thinner sections, which basically crushed the tissue. By the early 1830s, Purkinje was the first to appropriately slice/compress thin tissue sections to prepare specimens for acute microscopic examination. Swiss Swiss anatomist Wilhelm His (1831–1904) is generally acknowledged with the invention of the rotary microtome in 1865 - a mechanical device enabling very thin slices (sections) of tissue to enhance microscopic level study.

Embedding

German/Swiss microbiologist Theodor Albrecht Edwin Klebs (1834–1913), renowned predominantly for pioneering modern bacteriology and the study of infectious diseases (he identified the bacterium causing infection with diphtheria), introduced paraffin wax embedding around 1869.

Formaldehyde

In 1859, Russian chemist Alexander Mikhaylovich Butlerov (1828–1886) accidentally produced formaldehyde while synthesizing methylene glycol. In 1868, German chemist (originally a law student) August Wilhelm von Hofmann (1818–1892) conclusively identified and laid the foundation for the modern manufacturing process of formalin in preparation and fixation of soft tissues. German physicians Ferdinand Blum (1865–1959) and his father Isaac Blum (1833–1903) advocated the use of formaldehyde in laboratory medicine due to its effectiveness for maintaining the color and shape of tissues with no change to the microscopic structures. Although an essential improvement in both anatomical and histological laboratory studies, as the toxicity became more evident, formalin became (and remains so today) one of the most highly regulated safety concerns.

19th Century (1801–1900)

Along with the transformation to political, social, and industrial revolutions replacing societies previously dominated by religion and aristocracy, the advancements in the microscope, the introduction of standards of measurement, and knowledge about hematology, microbiology, immunology, histology, and chemistry paved the way for a number of significant and useful laboratory tests during the 19th century. The 1800s marked the beginning of the usefulness of newly evolved laboratory testing as specific chemical, bacteriological, and hematological diagnostic tools. Even the study of urine was rediscovered as urinalysis.

Pathology until this century still played no direct role in patient care. Most pathologists were segregated to teaching/research and performing autopsies. However, with advancements to the microscope. a new era of pathology and laboratory medicine as distinct disciplines evolved. The cross-training and multitasking involved in the various laboratory disciplines meant that each were better appreciated and understood for their contributions.

French professor Gabriel Andral (1797–1876) was a pioneer of blood pathology and was considered the "Founder of Clinical Hematology." He studied the blood of both animals and humans and was the first to describe the constituent proportions of normal blood. His opposition to bloodletting as a practice performed by many physicians was a direct result of noting that disease affects the blood. Referring to "pus" in the blood, in 1843 he detected an increased number of "globules" (which were WBCs) and a decreased number of "another globular element" (which were RBCs). Andral was essentially describing leukocytosis and anemia.

Simultaneously, English practitioner William Addison (1802–1881) reported in 1843 the first description of "white globules" (leukocytes), concluding that both known blood elements—RBCs and WBCs—were markedly altered in infections.

Curiously, almost 2 centuries passed from the discovery of RBCs to the discovery of WBCs, but one must consider that RBCs outnumber WBCs by millions to thousands per cubic millimeter and the transparency of WBCs.

French physician Alfred Francois Donne (1801–1878) is acknowledged for expanding the use of the microscope to all human body fluids, notably discovering the parasite *Trichomonas vaginalis.* In 1842, he reviewed the microscopic appearance of blood smears, noting the existence of a total of 3 blood elements: red "globules" (RBCs) and elevated white "globules" (WBCs) along with "little globules" (not yet named). By 1844, the excess of WBCs in sick patients convinced him to suspect the maturation arrest of intermediate cell development. Donne linked abnormal blood pathology for the first time in medical history to what would become known as leukemia. By 1845, he was the principal proponent of a new, cutting-edge technology: photography with microscopy.

Scottish physician and pathologist John Hughes Bennett (1812–1875), a student influenced by Donne, published a text on the use of the microscope for diagnostic purposes in 1841, defining it as a clinical instrument. He opposed bloodletting and supported the admission of women to medical schools. He also offered the first published description in 1845 of "leucocythemia" as a systemic blood disorder resulting from purulent "colorless corpuscles—leucocytes." He also detailed the pathogenic fungus in human lungs (*Aspergillus* sp).

Although there is much ado in medical history regarding the origin of platelets, several physicians share the credit for discovering them. Italian pathologist Giulio Bizzozero (1846–1901) is generally credited in 1882 with the microscopic comprehensive description of living animals having "little plates," having been inspired by German anatomist Max Schultze (1825–1874), who in 1865 came upon what he referred to as "spherules" that were smaller than RBCs and WBCs and sometimes clumped together. Notably, Schultze was educated in music and in medicine and preferred a laboratory research career over the practice of medicine.

German Prussian-born pathologist Rudolf Carl Virchow (1821–1902) was one of the most prominent "laboratory" physicians in the 19th century. He was the first to study disease from organs all the way down to the cellular level, using the microscope routinely in autopsies. He brought a scientific basis to medicine and pioneered the application of the cell theory, that all cells arise from pre-existing cells. He wrote more than 2000 scientific papers and coined more than 50 medical terms such as "thrombus," "embolism," and "chromatin." Although he erroneously dismissed the idea of the migration of leukocytes, in 1847 he detected reversed WBCs and RBCs, naming the blood condition "leukamie" originating from previously normal cells and thereby contributing to the theory of cancer. He has been coined the "Father of Modern Pathology."

Another strong advocate of microscopy was British scientist Lionel Smith Beale (1828–1906). In 1854, Beale reported on his cell classifications that were based on shape and body origin and in particular his descriptive discovery of the cell's nucleus and nucleolus. He also described sputum cancer cells in 1860.

But of all the extraordinary laboratory testing advances, it was German physician Paul Ehrlich (1854–1915) who probably made the most substantial discoveries for the most laboratory areas—histology, hematology, immunology (for which he received the Nobel Prize), microbiology, and chemistry—by inventing a repository of stains. He experimented progressively with aniline chemical dyes and in 1877 created his "triacid" stain, which enabled the detailed staining of tissue, bone marrow, and blood cells. Acidophils (now known as eosinophils), basophils, and neutrophils were clearly shown as were mast cells and nucleated RBCs. In fact, at age 25 Erlich published the technique of staining blood films for differential counting. Unfortunately, the pigment solution (Ehrlich's reagent), interrelating chemistry with the other laboratory disciplines in laboratory medicine, was rejected at the time because of the lack of chemistry knowledge in the medical profession. Modifications of the Ehrlich staining methods made by Russian physician Dimitri Leonidovich Romanowsky (1861–1921), German chemist/bacteriologist Gustav Giemsa (1867–1948) in 1905, and American pathologist James Homer Wright (1869–1928) in 1906 advanced blood film staining as it is utilized today. A laboratory medicine area also ascribed to Romanowsky for the general staining of cytopathologic specimens is now known as Cytology.

In 1887, Ehrlich's tuberculosis was confirmed using his own stain to identify tubercle bacilli in his own sputum. He also researched antitoxins and discovered the "autoantibodies against oneself" and attempted to find a chemical capable of killing the microbe but avoiding the rest of the body's healthy cells.

Erlich's stains distinguishing cells enabled differentiation and the identification of different hematological disorders, including anemia and leukemia. Although Ehrlich has been painted as an indifferent student who disliked formal exams and sometimes had an unpleasant relationship with medical leadership, his staining of blood films brought hematology into a new era and left an everlasting impression on Laboratory Medicine.

20th Century (1901–2000)

Two additional distinct clinical laboratories historically were not developed as disciplines in laboratory medicine until much later in timelines:

Blood Bank/Immunohematology

It was not until 1901 that Viennese pathologist Karl Landsteiner (1868–1943) introduced the modern blood

Lab Medicine 2021;52;e66–e82 e75
DOI: 10.1093/labmed/lmaa098

typing concept and described the ABO blood group classifications. He later contributed to the Rh factor discovery relating human blood to the rhesus monkey blood. Charles R. Drew (1904–1950) was a prominent African-American surgeon who worked closely with the first American Red Cross Blood Bank in 1941 - becoming the first director. He developed blood banks early in WWII saving thousands of Allied forces and shipped 'bloodmobiles' (trucks with refrigerators of stored blood) to mobilize quickly. He also utilized a cream separator devised by the British to separate plasma from RBCs and mass produce plasma.

Special Hematology - Coagulation

The blood coagulation advances by English physiologist William Hewson in 1770 did not proceed with any momentum. The classical theory mechanism for converting prothrombin using tissue (extrinsic pathway) continued but the blood clotting without tissue (intrinsic pathway) was not pursued for approximately another 40 years.

It was not until 1905 when Paul Morawitz (1879–1936) a 26 yr old German physician became the 'Founder of Classical Coagulation Theory' by discovering that tissue extracts did not coagulate fibrinogen and introduced the clotting theory. This laid the foundation of the coagulation cascade. And despite one of the most frequently ordered test for haemostatis - PT - was not developed until 1935 and APTT was not measured until 1953. Special hematology as we know it today was not a separate laboratory science until the 1950s when complex interactions and coagulation factors were studied and utilized.

Chemistry

This laboratory area requires another mention again due to noted particular advancements in the 20th centuries from simple qualitative to quantitative analysis of urine and blood.

Of particular mention is German pathologist Max Jaffe (1841–1911) who discovered the principle that color changes directly proportional to concentration - and introduced the Jaffe reaction in 1886 as a colorimetric method This analytical method remains one of the oldest methodologies still in use after 134 years.

But is wasn't until Swedish-born chemist Otto Knut Olof Folin (1867-1934) that groundbreaking advancements were noted. In 1882 he migrated as a 15 yr old to USA and by 1907 was appointed to Harvard Medical School. He was intrigued by the advancing area of chemistry and incorporated and adapted the Jaffe reaction from research - the first reaction of uric acid with picric acid. Even more remarkable - he was responsible for developing several methods for quantifying human waste produces in urine including creatinine, uric acid, urea, ammonia, creatine - and introduced the principles of protein metabolism. He was able to precipitate proteins in blood without adsorbing the non-protein.

From 1920, he collaborated with China-born biochemist Hsien Wu (1893–1959) to produce several micro methods for quantifying non-protein constituents suas as the first assay for blood sugar (Folin-Wu method). Many laboratorians consider this "American" the "Father of the subspeciality of Laboratory Medicine - Modern Clinical Chemistry."

The Earliest Laboratories

From Teaching and Research to Analysis

Initially, laboratories were used predominantly for teaching and research, which progressed to the study and investigation of public health diseases. With the great London cholera outbreak in 1854, English physician John Snow (1813–1858) studied water pumps as the source of contaminated water and analyzed the disease patterns, which can be seen as beginning the application of laboratory methods (chemical and microscopic examination of water) for patient safety.

This reliance on the laboratory to determine etiological agents continued with the groundbreaking work of Louis Pasteur (1822–1895). Pasteur discovered the existence of aerobic and anaerobic bacteria, the partial heat sterilization of microbes, and the vaccines against anthrax and rabies. A French biologist, microbiologist, and chemist and the "Father of Bacteriology," he was a noteworthy contributor with significant breakthroughs in sterilization, vaccination, and pasteurization. His work has saved countless lives via laboratory testing.

Robert Koch (1843–1910) was a brilliant bacteriologist and is considered the "Founder of Bacteriology." He advanced the culturing of microorganisms, establishing a direct relationship between microbe germs and principal infectious

diseases. In 1882 he isolated the tuberculosis bacillus pathogen responsible for numerous deaths in the 19th century, winning the Nobel Prize in 1905. He also identified the causative bacillus of anthrax and the bacterium of cholera.

Notably, Koch originally began growing bacterial colonies using slices of potato. However, because this was not a suitable medium for growth, he switched to another nutrient: gelatin.

In 1881, German microbiogist Walther Hesse (1846–1911), Koch's laboratory assistant, suggested agar as a favorable medium. It was his wife Fannie Hesse (1850–1934), working as an unpaid laboratory assistant, who discovered this phenomenal nutrient of the microbiology laboratory. She was using it at home to make puddings and jellies and became acutely aware of its properties maintained at heated temperatures. Some historians distinguish her as the 'Mother of Microbiology.'

In 1887, another assistant in the laboratory, German microbiologist Julius Richard Petri (1852–1921), invented the Petri dish. A substantive component of the microbiology laboratory, it prevented contamination and could slide under a microscope. This old laboratory tool remains the same and is still in use in modern microbiology laboratories.

Dutch microbiologist Martinus Willem Beijerinck (1851–1931) furthered the work of Edward Jenner and Louis Pasteur by developing vaccines as protection against infections, at a time when viruses were not yet identified. After studying the work of Russian botanist Dmitri Ivanovsky (1864–1920), who in 1892 demonstrated that there was 'something' not being filtered with bacteria from infected tobacco plants, Beijerinck called the infectious substance 'virus- beginning the field of virology.

Laboratory Equipment

What did the original clinical laboratories look like (**Figure 3**)? Where were the first clinical laboratories located? Any laboratories associated with teaching institutions were used purely for the purposes of experiments and for teaching. Early clinical laboratory procedures were performed by physicians within the confines of an office or home.

Although the various types of "laboratories" would be dependent on the designated testing being conducted—the laboratory techniques related to the procedures in use—the typical elementary laboratory room may have had some of the following key pieces of simple equipment or tools (depending on the nature of the study).

- Microscope
- Glass slides (originally pieces of ivory bone placed between the stage and the objective until the Royal Microscopical Society of London created the standardized 3 × 1 inch glass slides in 1840)
- Test tubes (invented by Swedish chemist Jons Jacob Berzelius in 1814 and refined by English chemist Michael Faraday in 1827)
- Beakers and flasks (invented by German chemist Emil Erlenmeyer in 1860). There has been some speculation that wine glasses were used before the invention of specialized glassware.
- Incubator (ancient Egyptian models that were used to keep chicken eggs warm were revised in the 16th century by Italian inventor Jean Baptiste Porta and in the 17th century by French scientist Rene-Antoine Ferchault de Reaumur. Notably, 19th-century practitioners predominantly relied on a simple bell jar that contained a single candle so that cultures could be placed near the flame on the underside of the jar's lid and placed in a dry heated oven.
- Scale or balance (invented by Richard Salter [England] in 1770)
- Bunsen burner (invented by Robert Bunsen [Germany] in 1855)
- Thermometer (invented by Daniel Gabriel Fahrenheit Germany in 1710)
- Petri dishes (invented by Julius Petri [Germany] in 1887)
- Forceps (invented by English Clergyman Stephen Hales who lacked formal medical training in the 18th century and originally used to remove urinary stones
- Tongs - (created by eldest son of the Chamberlen family of French surgeons who escaped religious persecution to England in the 16th century and originally used as an obstetric device for delivery of infants. The secrecy of this tool was not revealed until the 18th century.)
- Water Bath (used for heating and suspected to have been created by alchemist Maria the Jewess in 200 AD)

The laboratory "room" probably also had a table, a chair, and a stand - along with trays, lamps, and perhaps shelves for any chemical fixatives or stains in use.

DOI: 10.1093/labmed/lmaa098

Figure 3

The Earliest Laboratory

This was the simplest earliest laboratory, which is far different from today's highly modernized, automated, complex, and computerized instruments.

Two additional laboratory equipment of historical interest but which emerged much later in timeline are:

- Refrigerator (ancient Persians in 400 BC are credited by historians with the ice pit dome but not until 1748 with Scottish physician/inventor William Cullen was the first articial refrigeration system created. By 1805, US inventor Oliver Evans gave more practical uses for refrigeration and patents were granted in 1835 to American inventor Jacob Perkins who became the "Father of Refrigerator".
- Gloves (Not in use until the 1980s with John Hopkins' Hospital surgeon Dr. William Steward Halsted who created the disposable latex gloves as a regulatory compliance.)

In review, although initially the various early laboratories were developed for early scientists to perform experimental study and scientific research, none were specifically medically oriented for testing for direct diagnosis for patient care. Diagnostic laboratories would become the offspring to these early research facilities.

This summarized history of pathology and laboratory medicine is just the beginning. The next installment to be published discusses how pathology and laboratory medicine laboratories found their way into hospitals, who managed these facilities, and perhaps most importantly, who finally worked in these laboratories, and the development and emergence of the laboratory professional. **LM**

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Special Report

The Network That Never Sleeps

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Laboratory Medicine 2021;52:e83-e103

DOI: 10.1093/labmed/lmaa113

ABSTRACT

This review describes how Twitter is currently used by laboratory professionals for education, research, and networking. This platform has a global audience. It enables users to post information publicly, easily, rapidly, and free of charge. The absence of hierarchies enables interactions that may not be feasible offline. Laboratory professionals teach thousands of people using text, images, polls, and videos. Academic discussion flourishes without paywalls. Published research is shared faster than ever before, articles are discussed in online journal clubs, and research collaborations are facilitated. Pathologists network globally and make new friends within and beyond their specialty. Pathology departments and residency programs showcase trainees and faculty and celebrate graduations. As users in one time zone go to bed, others who are just waking up begin to read and tweet, creating a 24/7/365 live global online conference. We encourage others to plug into the power of Twitter, the network that never sleeps.

Keywords: social media, Twitter, professional development, education, networking, collaboration

You are at a laboratory medicine conference, mingling with your colleagues and friends from other states and countries. It's late in the evening. It's been a long and fruitful day of learning and connecting. You have presented your research and learned what others are doing. You have taught a course and attended a few others. Subject-matter experts were everywhere…it was so exciting! You connected with old colleagues, made new friends, and shared a laugh and a meal. Now it's time to go home.

Abbreviations:

CAP, College of American Pathologists; CME, continuing medical education; ASCP, American Society of Clinical Pathology; ISBT, International Society for Blood Transfusion; ASFA, American Society for Apheresis; USCAP, United States and Canadian Academy of Pathology; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; NRMP, National Resident Matching Program; HIPAA, Health Insurance Portability and Accountability Act.

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Now imagine that instead of the doors of the conference hall closing, they remain open. To everyone. Day and night.

As you go to bed, attendees in other time zones pour in. Everyone can see all the content created during the day: text, pictures, videos, comments, praise, and critiques. New attendees see what you and your colleagues said and discussed during the daytime, and they comment on it. They add their own teaching pearls and post their own takes on what transpired during the day. They network, mingle, debate, chat, and make new friends. This exchange goes on until they go to bed and you wake up in the morning and continue the cycle.

Now imagine that this conference is free. That anyone can attend any time without leaving their home or getting dressed up. That any trainee in any country can approach a famous professor and ask a question or strike up a conversation. Imagine that this conference goes on 24 hours a day, 7 days a week, 365 days a year.

This is the field of pathology on Twitter: the network that never sleeps.

Technology to Turbocharge Your Career

Twitter is best known as a social networking service (a type of social media), but this label does not do justice to the full range of ways in which the platform is used by medical professionals today. The somewhat flippant name of the platform contributes to the feeling among some people that its raison d'être is to post frivolous, humorous, or malicious one-liners.

From the perspective of medical professionals, however, Twitter is a free, easy-to-use, widely accessible 21st-century platform for global communication, a tool to "get the word out there" with unprecedented speed. It is also home to a diverse multinational community of pathologists and laboratory professionals engaged in constant information sharing, conversation, and 24/7/365 live real-time learning, free of traditional barriers that limit access and exclude many. This review—written by pathologists and laboratory professionals who use Twitter extensively for professional purposes—will describe the many ways in which Twitter is currently used by medical professionals in general and laboratory professionals in particular.

We remind the reader that the audience on Twitter is composed not only of pathologists and laboratory professionals but also pathologists' assistants, residents, fellows, medical students, surgeons, oncologists, pediatricians, emergency-room physicians, practitioners of virtually every other specialty in medicine, basic scientists and, of course, members of the public.

The view that Twitter is conducive to academic discourse is becoming increasingly mainstream. As the scientist Alice Soragni, PhD, and the gastrointestinal pathologist and pancreatic cancer researcher Anirban Maitra, MBBS, argue in the journal *Nature Reviews Cancer*, "direct, unrestricted access to the biomedical community at large, together with the possibility to ask questions and obtain feedback in real time, is one of the singular advantages to using this platform for communicating published or unpublished data."[1] Soragni and Maitra further state, "our professional networks have become larger and more diverse thanks to Twitter, and we are better for it."

We agree. Twitter has made our professional networks larger and more diverse as well, and in doing so, has turbocharged our careers. We hope we can show you how.

What Is Twitter?

Twitter is a microblogging platform or application (app) that allows users to post messages (information) in small portions known as *tweets* (≤280 characters), images (as many as 4 per tweet) and short videos (as long as 2 minutes, 20 seconds). A Twitter account is required for posting tweets but not for reading them. Tweets can be read by anyone with access to the internet and a web browser. This feature enables information to be rapidly made public and widely accessible.

Twitter is best known as a smartphone app but it can also be used on a tablet or in an internet browser on a desktop or laptop. It can be downloaded free of charge in most countries and includes a one-click translation feature to increase accessibility.

How to Get Started on Twitter

The process takes only a few minutes. You grab your cell phone, download the (free) Twitter app, and create a username and password. You are now ready to tweet. Even easier, you can like or retweet a post created by someone else (see the upcoming section on retweets). Once on Twitter, you can read a step-by step guide that describes what you can do on the platform.[2] We also highly recommend the tips provided in a free online guide created by Jerad Gardner, MD, a pathologist who was instrumental in bringing pathologists and laboratory professionals to the Twittersphere.[3]

To let others know who you are, we recommend that you add a picture to your profile and say something about yourself (for instance, "I am a cytopathologist from Utah" or even just "pathologist" or "pathology resident"). A professional headshot is ideal, but even a simple selfie is better than a profile without a picture. We recommend starting with a photograph and a one-line description of yourself, and later editing your profile to expand and improve it. Examples of strong Twitter profiles are shown in **Figure 1**. Readers should feel free to use these profiles as templates to construct their own.

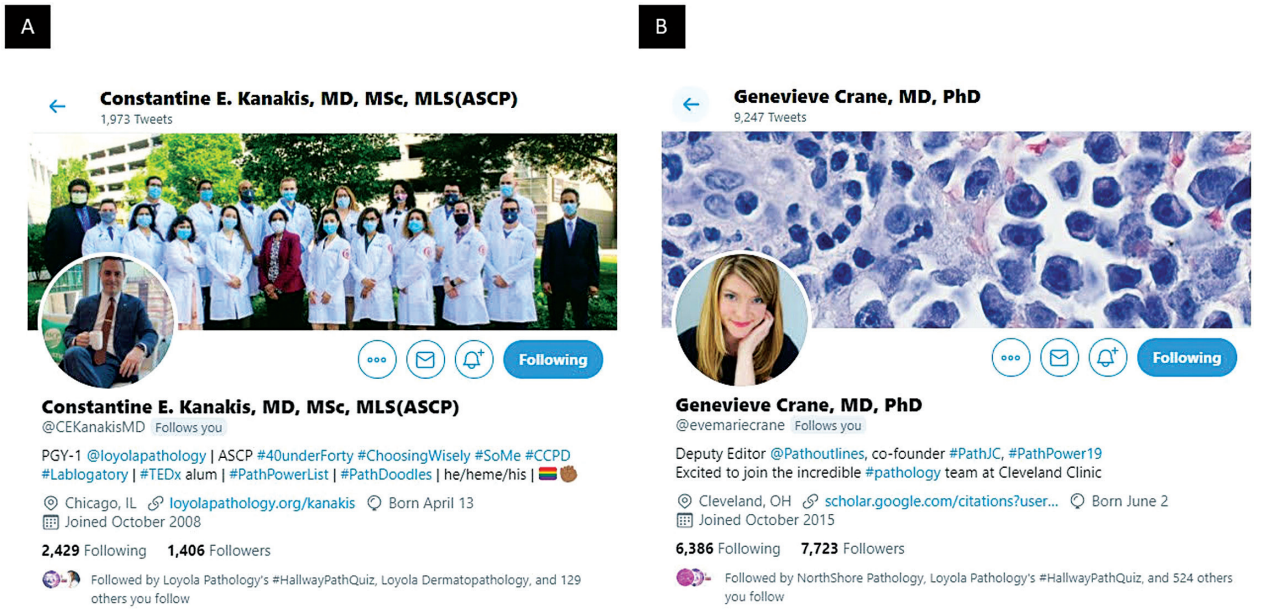

Figure 1

Examples of well-constructed Twitter profiles for medical/laboratory professionals. **A**, Twitter profile of pathology resident Constantine Kanakis, MD, MSc, MLS. **B**, Twitter profile of hematopathologist Eve Crane, MD, PhD. Both examples include profile and banner images, hashtags, location, and links to professional/institutional or academic profiles.

Do *not* leave your picture and Twitter bio blank. People like to know who they are following and are less likely to engage with an anonymous, faceless entity. The anonymity provided by an account without a real name or photograph is also ripe for misuse by trolls and bots.

It is possible to make your tweets private, but doing so would contradict the point of a public platform. It would be akin to walking onto a stage in front of a huge audience and turning your microphone off.

What Does It Mean to Follow Someone on Twitter?

Unlike in real life, where following a stranger would be creepy, following people on Twitter is essential to the architecture of the platform. Following is simply a courteous gesture or an indicator of interest in someone's tweets. When you follow someone on Twitter, their tweets appear in your timeline (*feed*). Likewise, your tweets appear in your followers' feeds. Most importantly, the reach of tweets is directly proportional to the number of followers.

But I Don't Know What a Hashtag or a Handle Is

A hashtag is a word preceded by a number symbol (#, also known as a hash or pound sign). It is a widely used, simple, and surprisingly powerful tool on Twitter. A hashtag can also precede a series of words without spaces, as in #learntousetwitter. Hashtags allow users to index tweets with labels that describe the subject or topic of the tweet. This enables tweets on any topic to be grouped together and easily retrieved in the future.[4] For example, you could type #pulmpath in the search bar on the Twitter app and read tweets on pulmonary pathology. Twitter users worldwide are constantly indexing their tweets with hashtags for future reference. **Table 1** lists a few commonly used

DOI: 10.1093/labmed/lmaa113

Table 1. Commonly Used Hashtags in Pathology and Laboratory Medicine

Hashtag	What the Hashtag Is Used For
Broad Topics	
#MedEd	Medical education
#pathology	Any topic in pathology
#PathTwitter	Any topic in pathology
#pathboards and #knoworfail	Pearls for the pathology boards
#pathbugs and #crittersontwitter	Microorganisms
Clinical Pathology	
#clinpath	Clinical pathology
#hemepath and #hematology	Hematopathology
#HumpDayHemepath	Weekly hematopathology tweet
#IDTwitter or #MicroRounds or #ASMClinMicro	Infectious disease/microbiology
#SHQuickByte	Pearls from the Society for Hematopathology
#Blooducation	Transfusion medicine and blood banking
#MicroMedEd	Tweets at the intersection of microbiology and medical education
Anatomic Pathology	
#surgpath or #SurgPath	Surgical pathology
#CytoPath or #FNAFriday	Cytopathology
#pulmpath	Pulmonary pathology
#GIpath or #gipath	Gastrointestinal pathology
#BSTPath	Bone and soft-tissue pathology
#liverpath	Liver pathology
#breastpath	Breast pathology
#GYNpath	Gynecologic pathology
#GUpath	Genitourinary pathology
#forensicpath	Forensic pathology
#neuropath	Neuropathology
#cvpath or #cardiacpath	Cardiovascular pathology
#pedipath	Pediatric pathology
#ENTpath	Head and neck pathology
#grosspath or #grossognosis	Gross (macroscopic) pathology
Miscellaneous	
#pathart	Art and pathology
#path2path	Discussion of pathology as a career
#mottirl	Met on Twitter, then in real life
#PathTweetAward	Tweets tagged as exemplary educational tweets
#PathRhymes	Tweets with rhymes about pathology diagnosis

hashtags in pathology and laboratory medicine. A list of pathology-specific hashtags has been published previously.[5]

Whether you want to add a hashtag to your tweet is entirely your choice. However, adding a hashtag helps broaden readership to those with similar interests and helps include those who may not have otherwise read the tweet. If we posted a tweet regarding this article, we might add hashtags like this: "We just wrote an article about Twitter for pathologists and laboratory professionals in *Laboratory Medicine*! #pathology #PathTwitter #MedLabTwitter." People searching for posts on #pathology would then discover this tweet.

Pathology residents navigating the overwhelming ocean of posts on Twitter are encouraged to try the hashtags #Pathboards and #PathTweetAward, which index tweets on pathology board examination pearls and tweets of educational value, respectively. For medical laboratory professionals, we recommend checking out the hashtags #medlabtwitter, #Lab4Life, and #MedLabChat. Tweets related to an individual conference or event can also be found easily if a hashtag is established, such as #ASCP2020 (for the American Society for Clinical Pathology 2020 Annual Meeting).

Handle is Twitter-speak for "name." Think of it as creating a pseudonym for yourself on Twitter. Although most professionals choose to make their real name visible on their brief Twitter profile, Twitter mandates that you create a Twitter-name that starts with the symbol "@." For example, Kamran Mirza, MD, PhD, goes by @KMirza. Any twitter user can tag Dr Mirza with his handle, which brings the tweet to his notice (via a notification) when he logs on to the app.

We are intrigued that the use of handles dispenses with some of the formality associated with interactions in professional life. Instead of addressing the first author of this article as Dr. Mukhopadhyay, you could call him @smlungpathguy on Twitter without causing offense, even if you are a resident or medical student. Instead of referring to him as Professor Deshpande, it is acceptable etiquette to address Professor Vikram Deshpande as @Vik_deshpandeMD. Although a handle can include part of a real name (eg, @CArnold_GI or @RunjanChetty or @feldstej), it isn't strictly necessary. Some users get quite creative with their handle, which can reflect their specialty, interests, origins, or other aspects of their life or personality. Examples include @IHC_guy, @Chucktowndoc, @infectio01, @rovingatuscap, @Mattcrophage, and @BldCancerDoc. To help facilitate interaction, Twitter handles can also be mentioned in the text of a tweet or tagged to an associated image or video without impacting the number of characters used for the tweet itself. This alerts others of your interest in their tweet, publication, or presentation, or indicates that you

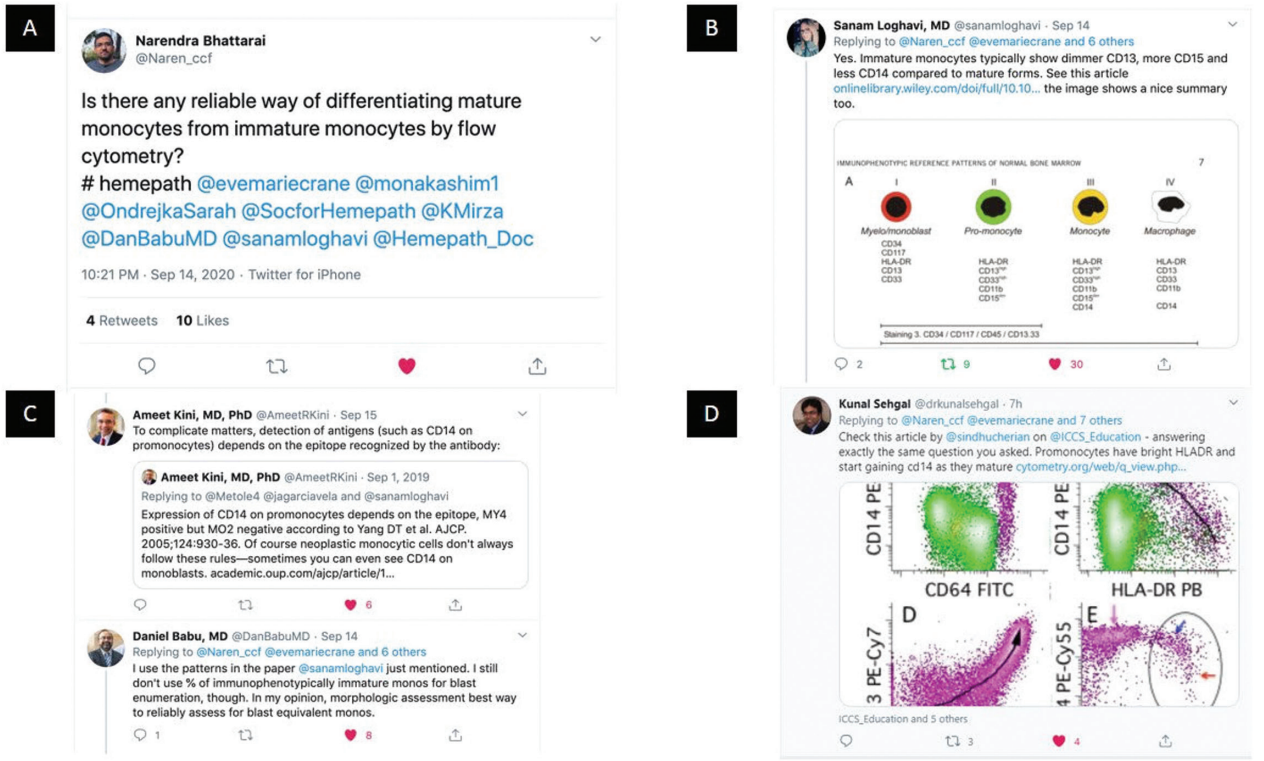

Figure 2

Tagging other Twitter accounts can enhance education and broaden networks. **A**, In this tweet, Narendra Bhattarai, MD (@Naren_ccf), a pathology resident at Cleveland Clinic, tags several hematopathologists on Twitter with a question that arose on his hematopathology rotation. This tweet yielded a concise and well-referenced answer from Sanam Loghavi, MD (**B**), sparking a conversation around that topic (**C**), with responses from Kunal Sehgal (**D**) and others.

wish to solicit feedback on a question or idea from those with expertise in the area (Figure 2).

Twitter's "Secret Sauce": The Retweet Button

The retweet button (symbol: 2 arrows curved in the shape of a rectangle) is a powerful tool that allows users to share posts tweeted by others with their own followers using a single click. It can be used in 2 ways.

One way is to simply click the retweet button without adding a comment. This method is quick and simple, and is a great way for newcomers to get started on Twitter without creating original content. Retweets build rapport because they amplify the reach of tweets, a gesture that is appreciated by the person posting the original tweet.

The other type of retweet (a *quote tweet*) not only reposts the original tweet but also adds a comment. Doing this takes more effort but is a great way to share a post and express one's opinion on it at the same time.

Education on Twitter

During the past few years, a robust body of literature[1–20] has emerged addressing various aspects of social media platforms in medicine in general, and pathology and laboratory medicine in particular. These articles cover a

Lab Medicine 2021;52;e83–e103 e87
DOI: 10.1093/labmed/lmaa113

range of issues surrounding the use of a variety of social-media platforms, including Twitter, Facebook, Instagram, YouTube, Periscope, Snapchat, and LinkedIn, among others. We will focus exclusively on Twitter because of the unique attributes that make it ideally suited for rapid, real-time global interaction and information sharing among medical professionals.

Tweets and Tweetorials

Anything you post on Twitter is a tweet. This could be a word, a sentence, a paragraph, an image, a GIF, a video, a poll, a link, or a combination of these elements. Twitter imposes a 280-character limit, but there is a simple trick to get around this: creating a thread or a Tweetorial.

A *Tweetorial* is a tutorial composed of tweets threaded into a linked sequence (thread). Tweetorials have become an increasingly popular teaching tool in pathology and laboratory medicine, as well as many other specialties in medicine.[4,6,21] A few examples of Tweetorials are provided in **Table 2**. The reader is encouraged to click the links provided and read a few Tweetorials, to get a sense of how they incorporate the tools that Twitter offers. The number of tweets in a thread is theoretically unlimited. The longest pathology Tweetorial currently contains 150 tweets.[22]

Twitter also has a poll feature that allows users to answer questions anonymously and compare their answers to those of others. Polls can be posted for a limited time period (chosen by the author) before the poll is closed and replaced by the final results. This tool is incredibly effective in increasing engagement and is occasionally used as a pretutorial test in Tweetorials to engage the reader, begin the discussion, and assess baseline knowledge. Some Tweetorials also feature a post-tutorial poll to gauge whether the Tweetorial increased understanding of key teaching points (**Figure 3**).[23]

The ability to share links within tweets is a key strength of Twitter and distinguishes it from Instagram, in which links are not clickable. This enables users to post links to published journal articles, other tweets, websites, and YouTube videos, facilitating dissemination of educational content.

As mentioned earlier herein, short videos can also be embedded within tweets. Innovative educators such as Karen Pinto, DNB, FRCPath (@TheKaren Pinto) and Tristan Rutland, MBBS (@TristanRutland7) have used videos within tweets to harness the full potential of Twitter as an educational medium.

Education in the Era of COVID-19 and Social Distancing

The role of Twitter as a platform for free global education in the era of social distancing has been recently described in detail.[4] Similar to other online platforms, Twitter was uniquely positioned to take on the challenges posed by the COVID-19 pandemic, which created an unprecedented demand for information related to SARS-CoV-2 (the coronavirus).

Table 2. Selected Twitter-Based Pathology Tutorials (Tweetorials)[a]

Author	Topic	Link to Tweetorial[a]
Matthew Cecchini, MD, PhD, FRCPC	Pulmonary Pathology: pulmonary amyloidosis	https://twitter.com/Path_Matt/status/1188506020554985480
BIDMC Infectious Diseases Fellowship	Infectious Disease: cystic echinococcosis	https://twitter.com/BIDMC_IDFellows/status/1298824845673795584
Rola Ali, MD	Giant cell-rich bone tumors	https://twitter.com/DrRolaAli/status/1217839247916523520
Luis Humberto Cruz, MD	Ganglioglioma	https://twitter.com/luishcruzc/status/1230867921603198976
Samson W. Fine, MD	Prostate anatomy	https://twitter.com/rovingatuscap/status/1156317771246002177
Valerie A. Fitzhugh, MD	Synovial sarcoma	https://twitter.com/DrFNA/status/1032086422327570432
Raul Gonzalez, MD	Hepatic masses	https://twitter.com/RaulSGonzalezMD/status/1237785642148302849
Daniela Hermelin, MD	Kell blood group	https://twitter.com/HermelinMD/status/1190014347193573376
Sanjay Mukhopadhyay, MD	Aspiration pneumonia (particulate matter aspiration)	https://twitter.com/smlungpathguy/status/1058922823857696768
	Immunotherapy	https://twitter.com/smlungpathguy/status/1236376706845421568
Karen Pinto, DNB, FRCPath	Testicular masses	https://twitter.com/TheKarenPinto/status/1223188799695532032
Avraham Z. Cooper, MD	Eosinophilic depletion s/p prednisone	https://twitter.com/AvrahamCooperMD/status/1304825763984703489
David Steensma, MD	Left-shift	https://twitter.com/DavidSteensma/status/1214387521104367618

[a]*All Tweetorials accessed on December 19, 2020.*

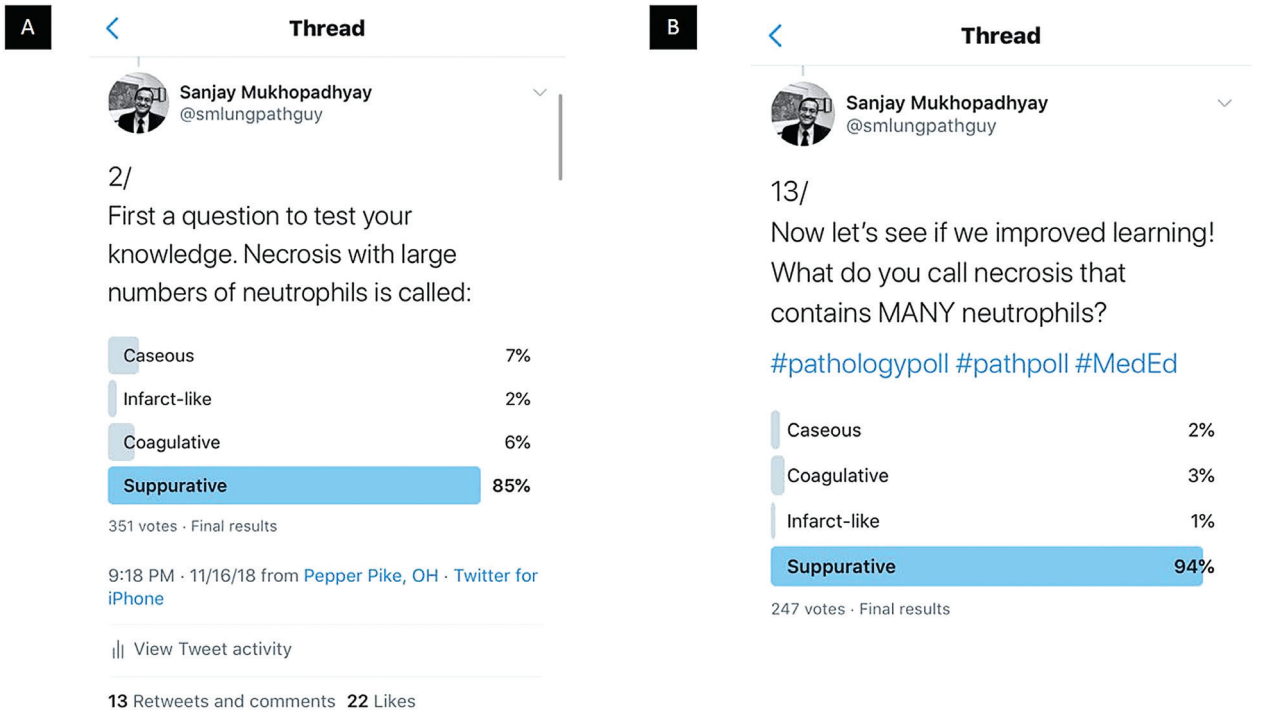

Figure 3

Polls can enhance engagement and facilitate assessment of learning. These tweets, part of a Twitter-based tutorial (*Tweetorial*), show an example of a pre-Tweetorial poll (**A**), which is easy to create. In the image shown, the poll is closed, and viewers can see the breakdown of answers. **B**, Answers to the same question after the Tweetorial. Note that the percentage of people who selected the correct answer increased from 85% to 94%.

The usual paradigm of academic publication, which emphasizes slow, careful review, often at the expense of speed, was upended by the seriousness and rapidity of events. Medical professionals responded by using this platform to rapidly share advances in the literature, add commentary, and provide context. Although the deluge of information that ensued had mixed results, some pathologists did an exemplary job in sharing breaking information virtually instantaneously while maintaining scientific integrity.[24] In a remarkable example of the power of Twitter to rapidly crowdsource post-publication peer review, commentary on the platform triggered an investigative effort that resulted in retraction of 2 dubious publications in the *Lancet* and the *New England Journal of Medicine*.[25,26]

Twitter was also used in synergy with other educational media during this period. For instance, live tweets were posted alongside a simulcast lecture by one of the authors of this review (C.K., @CEKanakisMD). The lecture was hosted by *PathCast*, an online compendium of pathology lectures. The topic of COVID-19 testing strategies and laboratory considerations registered 3900 views on YouTube; 17,100 views on Facebook Live; and 525 impressions from its initial Twitter post.[27]

#VirtualPath

Social distancing due to COVID-19 resulted in the rise of several remarkable ventures on social media united by the hashtag #VirtualPath.[4] Originally conceived as a label for all pathology-related activity on social media, #VirtualPath quickly morphed into virtual lectures, most prominently #CAPVirtualPath, a lecture series organized by the College of American Pathologists (CAP). Organization of this lecture series involved rapid recruitment of pathology and laboratory-medicine faculty, who agreed to give lectures that would be made freely available online by CAP. The lecture series became a massive success partly because it was promoted heavily on Twitter, attracting the attention of a large international audience that flocked to the lectures, encouraged by the unencumbered access to high-quality educational content. Christina Arnold, MD (@CArnold_GI)

Lab Medicine 2021;52;e83–e103
DOI: 10.1093/labmed/lmaa113

was awarded the 2020 CAP Distinguished Service Award for her role in organizing, promoting, recruiting for, and presenting for this lecture series.

Another project that started under the aegis of this hashtag was #VirtualPathGR (Virtual Pathology Grand Rounds: @VirtualPathGR on Twitter).[4] The Virtual Pathology Grand Rounds provides its audience with continuing medical education (CME) credits through the American Society of Clinical Pathology (ASCP). We also witnessed the inception of a virtual pathology student interest group (@Path_SIG).

Internationally recognized organizations and institutions joined this initiative by making their in-house lectures available on social media. One example was the posting of hematopathology content from MD Anderson Cancer Center using the hashtag #virtualhemepathMDA. These hashtags saw soaring engagement (measured by metrics provided by Symplur), with millions of impressions and hundreds of thousands of tweets.

#Blooducation

There has been tremendous growth on Twitter within the area of blood banking and transfusion medicine on a global level, thanks to the efforts of the AABB, the International Society for Blood Transfusion (ISBT), and the American Society for Apheresis (ASFA). These organizations encourage and promote educational tweets within these specialties, which often use the hashtag #Blooducation. The value of Twitter for promoting online education in these specialties is increasingly recognized.[28]

As in the world of anatomic pathology, Twitter-enabled collaborations in blood banking have taken many forms. Experts in the field with a Twitter presence are a valuable resource for laboratory professionals with questions about the standard of practice, in issues ranging from trauma resuscitation to discrepant findings on antibody panels. Blood banking technologists and specialists worldwide can provide real-time insights, advice, or additional resources when questions or challenges arise.

The speed of Twitter as a resource is illustrated by the responses to a tweet posing a question about the clinical significance of an anti-M antibody in pregnancy.[29] Within 60 seconds of the tweet, blood banking colleagues from around the world started sharing personal experiences and resources on this complex issue with a range of blood bank practices.

Another example of how Twitter is used in the blood-banking community is a tweet in which a question was posted in reference to a recently published article in the journal *Transfusion*, to gauge knowledge of the virtual platelet crossmatch.[30,31] Soon after the tweet was posted, members of the transfusion medicine community on Twitter replied, agreeing that this new technology has a role and noting that understanding how to implement it requires more information. AABB recognized this tweet as an educational opportunity, and the topic was chosen as the next journal article for the AABB Twitter-based Journal Club (#AABBjc), which took place in September 2020, generating tremendous engagement.[32] In addition to free online access to global participation, this virtual journal club also provides easy access for journal authors to join, lead, or even moderate the discussion. The September #AABBjc journal club discussion saw participation from 7 countries. An coauthor of the article (@KreuterMD) participated as a moderator.

Compared with traditional journal clubs, Twitter-based journal clubs achieve far more than highlighting the main points of an article. They provide an opportunity for a vast global audience to learn from colleagues around the world without geographic boundaries or paywalls, and to network while shedding light on knowledge gaps and needed areas of research. As an additional benefit, a multi-institution research collaboration that took root from this journal club will investigate the benefit of ABO-matched platelets in suspected platelet-refractory patients in the adult population. Twitter can also be used to raise public awareness about the importance of blood donation by recruiting donors for scheduled local blood drives or requesting urgent units for rare blood types.[33]

Finally, Twitter can be used to teach transfusion medicine topics beyond the traditional classroom setting. Tweetorials on these topics, freely accessible to any learner anywhere in the world, have been posted by us (D.H.).[34]

Twitter Homework

Even before social-distancing measures shut down in-person educational activities, social media platforms were experiencing a rise in endeavors seeking to promote pathology education. One such venture was #TwitterHomework, which took educational activities that

pathology faculty members had initiated in person in medical school pathology electives and brought them online to a global audience on Twitter.[35] This hashtag is used for a unique form of homework for medical students that encourages them to tweet about pathology-related topics. This practice can range from a retweet of a recent pathology post, to an original tweet featuring a pathology topic that the student recently learned about, to a post about their workday in pathology.

This activity encourages medical students to think for themselves, compose their thoughts on pathology-related topics into cogent posts, and learn from comments on their tweets made by others online. The exercise is helpful not only for the students but also for those viewing their tweets. #TwitterHomework has been well received, and participants report being pleasantly surprised at the warm welcome afforded to them by the pathology community in the Twitterverse.

PathElective.com

Pathology education became supercharged in the midst of the early months of COVID-19 with the development of a free, modular, interactive website curated by more than 50 volunteer pathology faculty members.[36] The website was conceived to bring many sources of pathology excellence together in one place and to mimic the process of an in-person pathology elective course. The website is divided into clinical pathology and anatomic pathology modules, with faculty members guiding students through different segments. Prequiz and postquiz assessments allow students to receive certificates of achievement.

Live Tweeting from Pathology and Laboratory-Medicine Conferences

Live tweeting refers to the practice of posting tweets from a medical conference while the conference is in progress. This practice enables those who cannot attend the conference in person to get a sense of the major developments occurring at the conference and to learn key take-home points.
In pathology, live tweeting was first popularized at the 2015 United States and Canadian Academy of Pathology (USCAP) Annual Meeting, where it was highlighted by the hashtag #InSituPathologists. Thanks to the intense interest generated online by this activity, the 2015 USCAP meeting went viral on Twitter and founded a social media movement that continues to this day.[37] Live tweeting from international pathology conferences is now routine, and metrics related to online engagement continue to increase.

#PathTweetAward

#PathTweetAward is a hashtag that encourages, recognizes, and rewards pathology tweets with exceptional educational value.[4,38] The hashtag was created in April 2018. Since then, it has been used by thousands of pathologists worldwide. The underlying concept is that any pathologist anywhere in the world should be able to nominate exemplary educational tweets. This nomination is accomplished by publicly flagging the tweet with this hashtag in a reply or retweet (**Figure 4**). A panel of screening judges searches for tweets containing the hashtag and compiles a list of tagged tweets that is posted on Twitter every week.[39] The screening judges then select the best educational tweets of the month and submit them at the end of the year to a separate panel of final judges. The final judges pick the 4 best educational tweets of the year, which are then entered into a Twitter poll open to the public. The poll results decides the order of the final winners, who are given a certificate and a small cash award.

In contrast to traditional awards, which are typically conferred by a few individuals behind closed doors and funded by pathology societies or large donations, #PathTweetAward is global, crowdsourced, and collaborative, with a transparent process that plays out online in full public view throughout the year. Anyone anywhere in the world can win, and everyone has a say in picking the winners. #PathTweetAward is funded by numerous small voluntary donations (*crowdfunding*).

Twitter for Research and Scholarly Work in Pathology

Is Anyone Reading My Paper? Disseminating Your Research

As things currently stand in academic publishing, authors seldom (if ever) learn whether anyone has read their published work. The only hard indicator is a citation by another published article. Many articles published in academic

Lab Medicine 2021;52;e83–e103 e91
DOI: 10.1093/labmed/lmaa113

Figure 4

How to use the hashtag #PathTweetAward on Twitter. **A**, Pathologist Tristan Rutland, MBBS, retweets a tweet by Mark Ong, MBChB, and tags it with the hashtag #PathTweetAward. This simple action labels the tweet as a nominee for the award and brings it to the attention of the screening judges. **B**, In this tweet, Cullen Lilley, an MD/MA student, retweets a tweet by microbiologist Aaron Odegard, PharmD, MS, and tags it with #PathTweetAward. Note the excellent use of images in both original tweets.

journals receive few or no citations, and it is entirely possible that many are read by few (if any) readers.

This lack of interest and engagement is mirrored by online activity measured by web-based services such as Altmetric. As shown by Altmetric data, most publications generate very little attention online, whether in the general public, in blogs, or the national news media. Journals with low impact factors and niche audiences are particularly susceptible to lackluster enthusiasm and underwhelming readership. In the past, an individual author could do little to change this dismal state of affairs.

Twitter has radically altered this paradigm.

Now, authors have a tool to publicize and showcase their scholarly work. Assuming that most authors want their work to be read widely, this is a welcome development. Authors can achieve this end in many ways, including posting a tweet or Tweetorial with a link to their article, discussing their findings with the medical community on Twitter, creating polls related to the article, and discussing their work in online journal clubs such as #PathJC.

Genuine enthusiasm for one's scholarly work, coupled with a desire to communicate the work to others, stimulate discussion, and solicit feedback, is welcomed by the pathology community on Twitter. Discussion of articles online provides an opportunity for authors to gain perspective on their research by reading comments from a large number and variety of professionals from all over the world, some of whom may be experts in the field.

Figure 5 illustrates the impact of a tweet about an article. This tweet by the Society for Hematopathology (**Figure 5A**) contains a link to an article published in the *American Journal of Clinical Pathology*. An image from the article helps to enhance interest and draw in readers. **Figure 5B** shows analytics for the tweet, available on the smartphone of the person who composed it. The tweet shown here generated

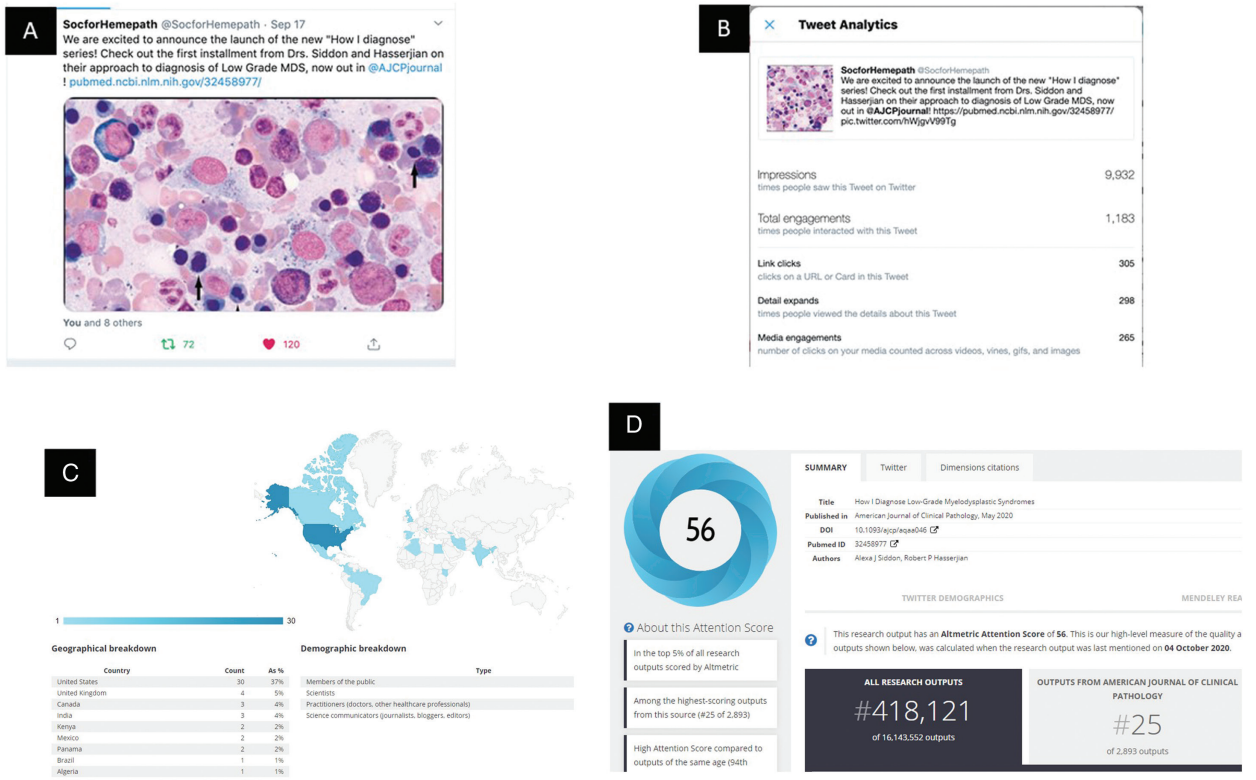

Figure 5

Promotion of manuscripts on Twitter can enhance engagement. **A**, A tweet from the Society of Hematopathology (@SocforHemepath). **B**, This tweet generated more than 300 clicks on the link to a related article. **C**, Geographic and demographic breakdown of people who engaged with the tweet. **D**, This tweet helped contribute to an Altmetric score in the top 5% of all research outputs. Metrics for the potential impact of a tweet can be readily tracked.

9932 impressions (potential viewers) and 1183 engagements (people who liked, retweeted, replied, clicked on the link, or clicked on the image). Today, many journals and publishing houses collect and closely monitor metrics on the online impact of their articles, via tools such as Altmetric or PlumX. **Figure 5C** shows the countries where users were tweeting about the article and **Figure 5D** provides additional metrics, including the fact that the Altmetric score of this article (namely, 56) was ranked number 25 of more than 2800 articles followed for impact from this journal.

In the modern era, academic journals routinely track and showcase their most attention-generating articles (**Figure 6**). The degree of activity generated on Twitter is a contributor to the Altmetric score, which also takes into account mentions of the article on a curated list of Facebook pages, Wikipedia, blogs, and patents. Mentions in the national and international news media are weighted heavily in the scoring algorithm.

Research Collaborations and Scholarly Work Facilitated by Twitter

In recent years, Twitter has been used in novel ways to promote scholarly collaboration. Such collaborations have resulted in several publications in peer-reviewed journals and even a few textbooks.[1,4,9,40–50]

In many instances, pathologists have collaborated on manuscripts in which the coauthors have only met online.[1,40] Early in the COVID-19 pandemic, Oklahoma forensic pathologist Lisa Barton, MD, PhD (@LMBarton1) reached out on Twitter to one of us (S.M.) based partly on his Twitter presence and his numerous lung-pathology tweets. Dr Barton was seeking an expert pulmonary-pathology opinion on 2 COVID-19 cases, which morphed into a collaboration that produced the first published article in the English-language peer-reviewed literature on COVID-19 in complete

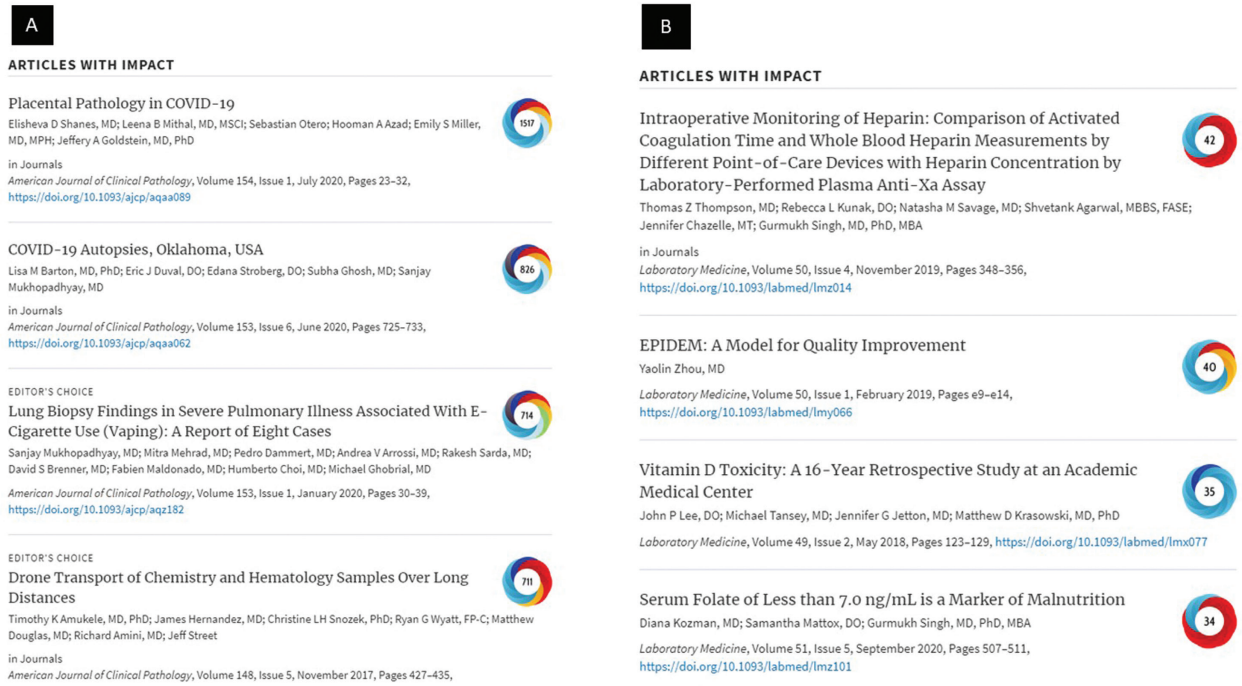

Figure 6

Academic journals use web-based scores such as Altmetric to assess impact, online attention, and readership with objective metrics. **A**, High-impact articles from the *American Journal of Clinical Pathology*. **B**, Altmetric scores for high-impact articles published in *Laboratory Medicine*.

autopsies.[40] The article generated intense interest, including coverage from the venerable magazine *Scientific American*, and has been highly cited in the literature (458 citations in 9 months per Google Scholar, as of February 2. 2021). Its Altmetric score (827) is the second highest of all time for the *American Journal of Clinical Pathology*. Additional published manuscripts also resulted from this online collaboration; to this day, the coauthors have never met in person.

In another example of the power of Twitter to rapidly create research partnerships, a tweet calling for lung-biopsy cases of vaping-related lung injury[41] led to a multi-institution collaboration that resulted in a publication on this topic. This article was also highly cited, had a high Altmetric score, and remains to this day as 1 of only 2 pathologic series on this entity,[42] to our knowledge.

The remarkable story of #EBUSTwitter has been previously described by Lepe and colleagues.[43] This hashtag was used for what is likely the first Twitter-enabled scholarly work in which a research idea was sparked by a tweet, fueled by another tweet, and executed almost entirely on Twitter, resulting in a peer-reviewed publication in the *American Journal of Surgical Pathology*[44] on a novel biologic phenomenon.

Twitter is especially well-suited for recruiting coauthors for review articles because so many experts use this platform. Potential collaborators often seek out others who write well (which is easy to judge on Twitter), work well together, and are interested in similar topics. We are aware of several published review articles[1,4,8,10,13,45] in which the coauthors met on Twitter. Currently, journal editors also have a Twitter presence and are aware of the excellent educational content being posted online. Occasionally, a high-quality tweet or Tweetorial will catch the attention of an astute, Twitter-savvy editor, resulting in an invitation to write a review article for an academic journal.[45,48]

Networking and Building a Community

Connecting With Other Professionals in Pathology

A major difference between Twitter and Facebook is that Twitter encourages interactions between individuals who may not be friends in the traditional social sense. For example, for a pathologist to appreciate or comment on an image of a fungus on Twitter, they do not need to befriend the person that posted the image. They can simply reply to the post, or like or retweet it. Because of this openness and ease of access, one can interact with virtually anyone on the platform, and they can choose to reciprocate if they wish. This structure fosters interaction between individuals who may have never met in real life, or would never have met given geographical boundaries or professional interests, or may never have approached each other. Another benefit of this environment is that it bridges gaps in seniority and experience to allow members of different experience levels to interact freely. A medical student or first-year pathology resident can easily ask a question addressed to a senior professor without the formality or barriers associated with traditional offline interactions.

A seldom-discussed variation on this theme is that Twitter is exceptionally well-suited to individuals who might be sidelined or disadvantaged in offline life due to their appearance, accent, shyness, or level of social skills. Unencumbered by these obstacles, such individuals can express themselves more freely on Twitter than they would in traditional settings. If one is to follow the adage to not judge a book by its cover, what better way than to read the book without looking at the cover?

#MOTTIRL

The hashtag #mottirl ("*m*et *o*n *T*witter, *t*hen *i*n *r*eal *l*ife") captures the phenomenon of professional relationships that start online and turn into real-life meetings and lasting friendships.[19] This situation typically occurs at national or international conferences, where individuals who have met, interacted, and known each other for months or even years online (but never offline) meet in person for the first time and experience the pleasure of meeting an old friend. This phenomenon is clearly a reflection of the smartphone era and the ubiquity and power of online social interactions. Individuals who meet in this way often tweet a selfie of themselves with the hashtag #mottirl to celebrate the moment they went from interacting strictly on social media to having offline interactions. Such moments cement genuine friendships and foster professional connections in a way unique to our digital generation.

Connecting With Professionals in Other Medical Specialties

Twitter makes it easy to expand one's network beyond the narrow confines of one's own specialty. Offline, increasing subspecialization frequently creates silos, in which people interact mainly with those in their own field, read journals catering to their own specialty, and attend national conferences tailored to the needs of a niche audience. In such an environment, it is unlikely that a pathologist will be well acquainted with the latest breaking news in, for instance, oncology, or will chat freely or frequently with oncologists. Is it equally unlikely that oncologists will be familiar with recent advances or areas of controversy in pathology.

Twitter breaks these silos by bringing everyone together on a common platform, where everyone can read everyone else's tweets in free full text and follow anyone in any specialty. On Twitter, our clinical colleagues frequently read our posts, debates, and viewpoints, and vice versa. Updates from clinical conferences rapidly spread to the pathology world. Good teachers and facile communicators develop sizable followings and become sought after as speakers and coauthors by specialists in other fields.

The authors of this article have made scores of such connections with professionals in other fields, leading to valuable professional opportunities such as invited lectures, speaking opportunities at national conferences, podcast invitations, and invitations to coauthor scholarly publications.

Freedom of Expression

The audience on Twitter greatly values freedom of speech and authenticity. In a world where messaging is increasingly controlled by corporate interests and vetted by handlers,

Lab Medicine 2021;52;e83–e103 e95
DOI: 10.1093/labmed/lmaa113

Twitter allows relatively unfiltered expression of the viewpoints of regular people.

Many laboratory professionals on social media celebrate personal events such as weddings and births, and share their hobbies and interests with their peers on social media. The hashtag #ILookLikeAPathologist is often used when pathologists share their interests outside of work. Members of the social-media community often join in to enjoy, congratulate, and celebrate, building a sense of community, positivity, and acceptance.

Whether you wish to share such information is entirely up to you. Many professionals prefer to keep their Twitter accounts entirely dedicated to professional purposes, whereas others prefer a blend of professional and personal purposes.

Supporting and Encouraging Others

The presence of a large group of professionals on a shared platform opens up the opportunity for mentor-mentee relationships. Finding mentors can be difficult if one is working independently, but informal mentorships are common across social media. For experienced practitioners who are social-media savvy, Twitter can be a great place to offer wisdom and career advice to younger professionals starting out in the field. Many pathologists serve as role models for others on this platform by virtue of their positive attitude and their willingness to teach.

Reaching out to a potential mentor can be as simple as tagging them in a tweet. For instance, a junior pathologist might put out a short tweet about her research or a case that she published and tag an experienced pathologist in the tweet. This practice results in instant exposure (the tagged individual might read the paper and reply with a comment) and opens up the possibility of an informal mentoring relationship.

Another way in which Twitter is used to support and encourage others is via online celebrations, which have become common during the era of COVID-19. In early 2020, a number of medical schools canceled all in-person learning and went fully online. This included cancellation of Match Day celebrations, which are a beloved tradition in medical school. To compensate, many schools started a "Virtual Match" celebration, in which students held a Zoom meeting or a YouTube live stream to announce where students in their class had matched. This activity soon moved onto Twitter, where a number of #virtual match tags were created, including some for specific fields. In this way, members of a field could congratulate incoming members and show support for an exciting life event.

Other significant life moments and professional accomplishments are also commonly celebrated on Twitter. Pathologists and laboratory professionals post about fellowships, awards, promotions, program acceptances, dissertation defenses, invited lectures, and moves to new institutions. The Twitter pathology community welcomes such posts in a collegial manner and serves as a source of constant encouragement and bonhomie. It is tempting to speculate that belonging to a welcoming community of one's peers might reduce burnout for some individuals.

Making positive career developments public has an added benefit. It makes a vast audience (including potential future bosses or colleagues) familiar with one's achievements and career trajectory. In this way, Twitter can function as a continuously updated curriculum vitae that is open to the public.

Group Chats

One method of building a community on Twitter is a *group chat* (**Figure 7**), a question-and-answer session that allows individuals to discuss issues related to their area of interest, expertise, or specialty. These chats encourage conversation and help to flesh out new ideas and collaborations. An example of a group chat pertinent to laboratory medicine is #MedLabChat, started in 2019 by Maria Roussakis, MLT, MSc (@MedLabMaria), Rodney E. Rohde, PhD (@RodneyRohde), and Contantine Kanakis, MD (@CEKanakisMD). #MedLabChat is a monthly group chat for medical-laboratory professionals that involves a minimum of 4 questions about a specific topic such as professional development, interdisciplinary interactions, nuances of certification, and licensure across various medical laboratory professional roles and continuing education.

Another example is #PathChat, which is tailored a bit more to pathologists. The most recent #PathChat discussed coronavirus testing methodologies, supply chain constraints, and testing platforms. Pathologists, medical microbiologists, and others participated and shared laboratory testing ideas, frustrations, and solutions. #CAPChat is similar to #PathChat but is run by the CAP (@Pathologists). #PathChat

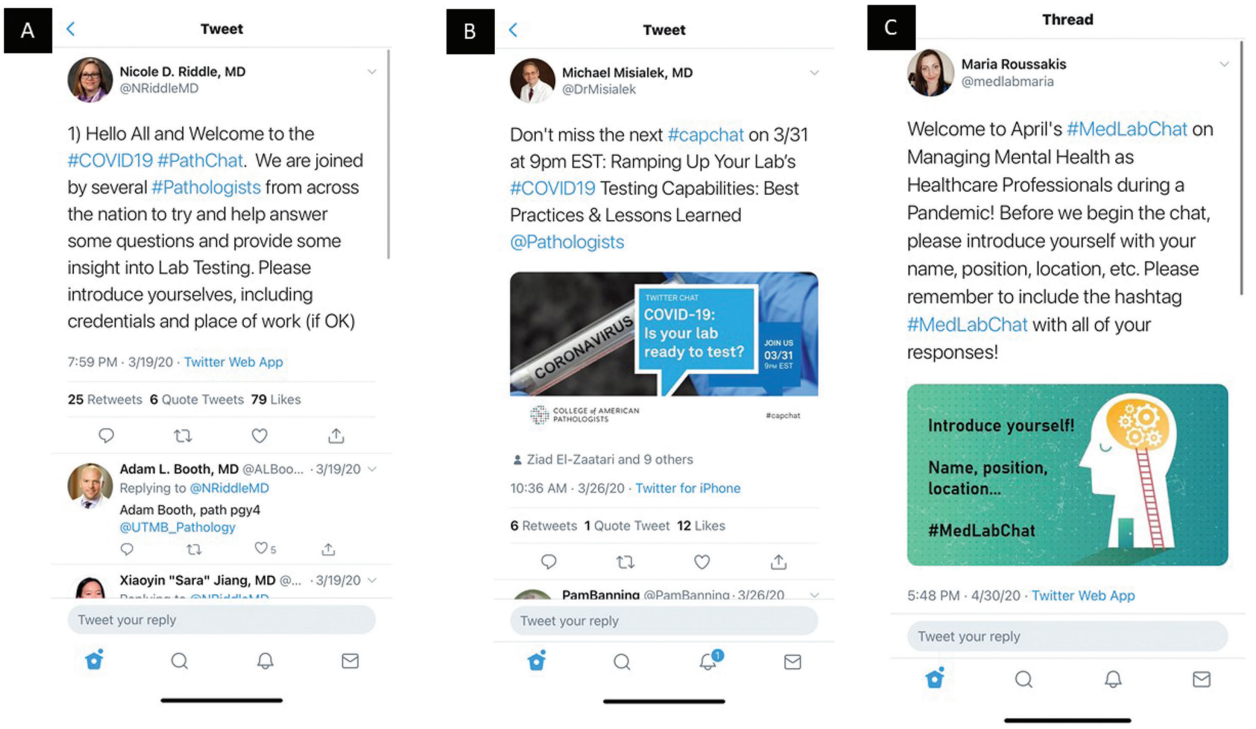

Figure 7

Examples of tweets for different laboratory medicine–themed chats. **A**, A tweet from Nicole Riddle, MD (@NRiddleMD), asking the first question for #PathChat. **B**, Michael Misialek, MD (@DrMisialek) announcing the next #CAPChat topic on COVID-19 testing capabilities. **C**, A tweet from Maria Roussakis, MLT, MSc (@MedLabMaria), welcoming participants to the #MedLabChat for April 2020.

and #CAPChat are moderated by members of the pathology community, who ask questions to initiate a discussion about a selected topic. The bulk of the discussion takes place during the hour that the chat is slated to occur, but discussions often continue on Twitter through the next couple of days as others log in and add their viewpoints.

Pathology Journal Clubs

A variation on a Twitter group chat is a Twitter-based online pathology journal club.[8,51] An early example of a Twitter journal club was the online nephrology Twitter journal club #NephJC, created in April 2014 to stimulate public discussion on current literature in nephrology, hypertension, and related topics across levels of training and specialties.[52] The excitement generated around these discussions was noted by a group of pathologists active on Twitter who—with mentoring from Matthew Sparks, MD (@Nephro_Sparks), of #NephJC—launched the first pathology Twitter-based journal club, with the hashtag #PathJC.[53,54]

Topics of discussion for #PathJC rotate across subspecialties, with approximately 1 topic per month. The journal club usually focuses on a recent paper with implications for diagnostic practice, such as the release of guidelines for diagnosis of acute leukemia or revised nomenclature of noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP).[55,56] These and others topics have garnered broad interest from pathologists in academia and private practice, pathology trainees, clinicians, and researchers. A #PathJC discussion on vaping-induced lung injury stimulated a robust and productive conversation that included members of the public, including the vaping community.[42]

Given the challenges of finding a single time that works across all time zones around the world, #pathJC expanded the

Lab Medicine 2021;52;e83–e103 e97
DOI: 10.1093/labmed/lmaa113

discussion to continue in a second time slot, adding the hashtag #eurotime to continue the conversation in Europe. These discussions typically generate impressions in the millions, provide a network for ongoing learning across practice settings, and spark collaborative research projects. In some cases, as exemplified by the vaping study, they also help raise public awareness of the role of pathologists in health care. The utility and impact of online journal clubs is increasingly recognized, and journals like *Modern Pathology* have granted full-text access to articles surrounding these events, to increase engagement.

Multiple subspecialty Twitter journal clubs within pathology have now been launched, including journal clubs focusing on dermatopathology (#dermpathJC), hematopathology (#hemepathJC), and gastrointestinal pathology (#GIPathJC). Setting these up is easy: all that is required is to create a hashtag and to alert potentially interested parties of when the event will occur. The #NephJC team provides a primer on their website for establishing such a journal club.[57]

Twitter for Pathology Departments and Pathology Residency and Fellowship Programs

Because in-person visits to pathology departments have decreased dramatically during the COVID-19 pandemic, establishing an online presence has become increasingly important. **Table 3** lists selected pathology departments and residency programs with a Twitter presence, and shows some of the ways in which these accounts post information in line with their mission.

These ways include showcasing the expertise, diversity, and personalities of faculty; highlighting the breadth of cases seen; and spotlighting the diversity and academic productivity of residents and fellows. Short videos featuring faculty are an excellent, no-cost, high-impact way to advertise the quality of one's department to a large audience.[58]

Such posts commonly reach thousands and help shape public perception of academic departments. As a result, these posts can potentially facilitate recruitment because they show trainees and junior faculty that your department is a supportive and productive place to build a career.

With cancellation of all National Resident Matching Program (NRMP) match celebrations across the country, the Twitterverse brought forward a virtual pathology Match celebration using the hashtag #VirtualPathMatch. With more than 4.4 million impressions and more than 1000 tweets in less than 1 month (**Figure 8**), this hashtag celebrated individuals involved in the Match process in 2020 and became the go-to mode for promoting and celebrating colleagues and friends.

We are now living in an era in which residency applicants are increasingly familiar with faculty members who have a robust online presence. Conversely, faculty members are increasingly aware of medical students who demonstrate enthusiasm for pathology on Twitter. The residency interview season is full of opportunities for applicants and programs to connect online on Twitter. Using a public stage like Twitter to highlight an applicant's interest, enthusiasm, or experience in the field may be potentially advantageous during interview season.

A Word of Caution

As with other technologies that have disrupted previous modes of communication—computers, the internet, and smartphones—Twitter can be used in productive and destructive ways. The potential hazards of social media use are well documented. Cell phone addiction is pervasive. The potential for misinformation and harassment exists.

There are understandable concerns about scientific integrity and expertise on Twitter. Because anyone can post freely on the platform without any type of vetting, the responsibility of deciding whether a person posting a tweet is a reliable source belongs to the reader. This situation opens up the possibility of dissemination of inaccurate or misleading information. However, because so many content experts are on the platform, fact-checking constantly occurs, and inaccurate information can be effectively debunked. We urge readers to be appropriately skeptical and (whenever possible) to cross-check published sources in the peer-reviewed literature. *Caveat emptor*.

Concerns about patient privacy are important and have been addressed in detail by Crane and Gardner.[7] Legal concerns are also understandable, but as Gardner and Allen[18]

Table 3. Examples of Pathology Departments and Residency Programs on Twitter

Pathology Department or Residency Program	Handle	Following	Followers[a]	Examples of Content Posted (Tweets)
The University of Michigan Department of Pathology: Ann Arbor, MI	@UMichPath	2214	7501	Educational cases, faculty publications, showcasing research, short profiles of current residents
Mayo Clinic: Rochester, MN	@MayoClinicPath	977	6976	Showcases of resident posters at USCAP meeting
Stanford Medicine Department of Pathology: Stanford, CA	@StanfordPath	97	5391	News and interesting cases
Department of Pathology at University of Arkansas for Medical Sciences: Little Rock, AR	@PathologyUAMS @HemepathUAMS	1444 926	4593 1976	Profiles, cases, professional development/promotions
Massachusetts General Hospital: Boston, MA	@MGHPathology	220	3907	Mixed features of faculty staff, accolades, cases, content
University of Alabama at Birmingham, School of Medicine, Department of Pathology: Birmingham, AL	@UABPathology	1436	3456	News, interesting cases, faculty/trainee profiles
State University of New York Upstate Medical University: Syracuse, NY,	@SUNYUpstatePath	4774	2716	Pictures of residents and fellows, resident interviews, short interviews with faculty
Anatomic and Clinical Pathology Program at the University of Texas, Medical Branch; Galveston, TX	@UTMB_Pathology	531	2491	Program promotion, cases, news
Anatomic and Clinical Pathology Program at Northwestern University, Feinberg School of Medicine; Chicago, IL	@NU_Pathology @NU_Glpath	408 151	2438 1089	Resident sharing, cases, professional development
Loyola University Medical Center; Chicago, IL	@loyolapathology @loyoladermpath @loyolahemepath @hallwaypathquiz @MLSatLoyola	779 238 84 176 72	2272 320 718 303 507	Educational cases, tweets promoting pathology education, tweets promoting training programs
Memorial Sloan-Kettering Cancer Center Department of Pathology; New York, NY	@MSKPathology	1	2129	Educational cases, promotion, projects
University of Chicago Department of Pathology; Chicago, IL	@UChicagoPath	244	1421	Content provided by residents, who run the account
Department of Pathology at Mount Sinai West and Mount Sinai Morningside hospitals; New York, NY	@MSWPathology	247	997	News and interesting cases
Ohio State Pathology Residency; Columbus, OH	@OSUPathRes	746	905	Interesting cases, faculty/resident profiles, news
Cleveland Clinic Pathology Residency Program; Cleveland, OH	@CCFPathRes	77	609	Faculty interviews, brief resident profiles, showcasing of teaching techniques and diversity
Medical College of Wisconsin; Milwaukee, WI	@MCWPathology	77	491	Department accomplishments, publications, profiles, cases
NorthShore Pathology Residency Program; Evanston, IL	@NorthShore_Path	349	313	Profiles, cases, interdepartmental news sharing
Geisinger Pathology Residency Program; Danville, PA	@GeisingerPath	27	278	Educational material posted by faculty, pictures of residents, link to residency-program website
University of Washington Pathology Residency and Fellowship; Seattle, WA	@UWLMPathRes	39	266	Feature profiles, publications/articles, case reports
University of Arizona Pathology; Tucson, AZ	@UAzPathology	43	221	Cases, profiles, Arizona features, news, accomplishments

USCAP, United States & Canadian Academy of Pathology.
[a]*Follower counts change constantly. These numbers are accurate as of September 27, 2020.*

DOI: 10.1093/labmed/lmaa113

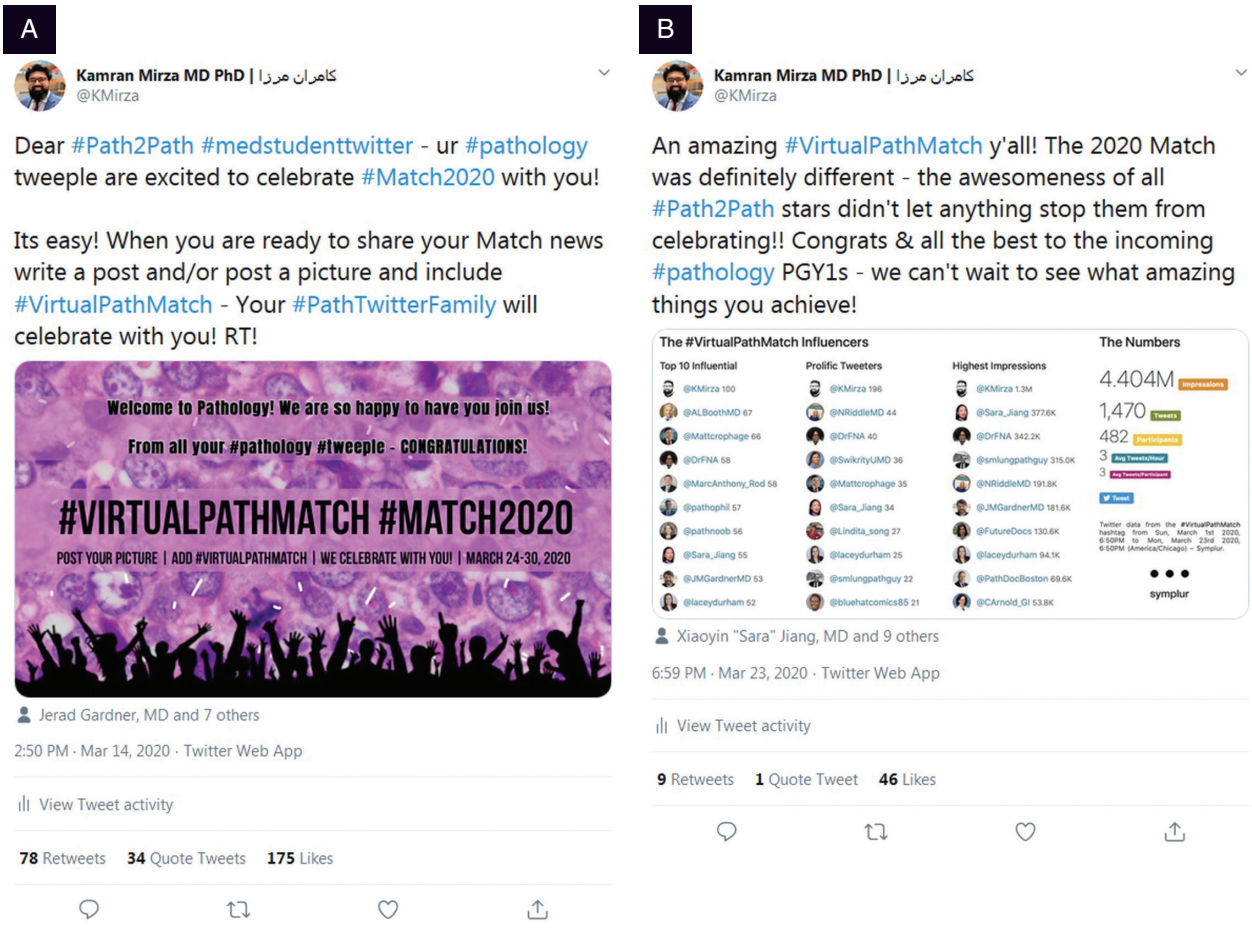

Figure 8

#VirtualPathMatch. Tweets marking the beginning (**A**) and end (**B**) of #VirtualPathMatch, a Twitter-based celebration of the National Resident Matching Program (NRMP) Pathology Match 2019. The hashtag, created by Kamran Mirza, MD, PhD on March 14, 2020 generated more than 4.4 million impressions during a 9-day period (data shown March 1 through March 23, 2020 - the 9-day period noted is between March 14 when the hashtag was created and Match Day, March 23).

have pointed out, no lawsuits have been filed thus far based on misuse of social media by pathologists.

We urge everyone using Twitter and other social media platforms to never post identifiable patient information. We urge them to be mindful of Health Insurance Portability and Accountability Act (HIPAA) regulations and, above all, to strive to be supportive, kind, and collegial.

Remember that all tweets are public. Minimize clinical information in posts about cases. The time of the post, combined with location data, can make data identifiable, particularly if a tweet is related to an unusual or rare entity.

An important principle in educational pathology posts is to focus on the teaching point and to remove information that is not critical to the message.

Tweet the lesson, not the patient.

The social media policies enforced by your institution may be more restrictive than HIPAA regulations. Adherence to these policies is always advisable. This practice is especially important for officially sanctioned departmental, residency, or fellowship accounts, which are more likely to be viewed as representing the organization.

DOS
Post educational tweets and create tweetorials using all the tools Twitter has to offer (text, images, video, gifs, polls)
Post links to articles or books you have authored or read
Encourage others who tweet good educational content
Promote good conferences or speakers
Introduce new members to the online pathology community
Share pearls from good published articles (always cite the source!)
Attend Twitter journal clubs
Communicate and engage with others on the platform
Ask questions and engage in productive debate
Showcase your trainees, colleagues, friends, department and training programs
Make friends across specialties, cultures and countries
DON'TS
Don't post identifiable patient information
Don't call people names
Don't harass anyone in any way
Don't get into fights if people are rude or disrespectful to you (your options include muting, ignoring and blocking)
Don't post copyrighted material
Don't post any content (text or images) posted by someone else without citing the author
Don't post anything you wouldn't want your boss, family or patients to see

Figure 9

Advice for Laboratory Professionals on Twitter

Excellent guides for newcomers including Twitter dos and don'ts have been previously published.[59,60] We summarize our advice in Figure 9.[2]

Summary

Twitter is a modern communication tool that puts the ability to post public messages with unprecedented speed and reach into the hands of the common person. For those in the medical professions, this tool opens up innumerable opportunities for education, research, and networking, some of which we have described in this article. We urge all laboratory professionals to take the plunge and experience first-hand the power of Twitter, the network that never sleeps. LM

Personal and Professional Conflicts of Interest

None disclosed.

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DOI: 10.1093/labmed/lmaa113

Laboratory Predictors of COVID-19 Pneumonia in Patients with Mild to Moderate Symptoms

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Laboratory Medicine 2021;52:e104-e114

DOI: 10.1093/labmed/lmab015

ABSTRACT

Objective: This research aims to develop a laboratory model that can accurately distinguish pneumonia from nonpneumonia in patients with COVID-19 and to identify potential protective factors against lung infection.

Methods: We recruited 50 patients diagnosed with COVID-19 infection with or without pneumonia. We selected candidate predictors through group comparison and punitive least absolute shrinkage and selection operator (LASSO) analysis. A stepwise logistic regression model was used to distinguish patients with and without pneumonia. Finally, we used a decision-tree method and randomly selected 50% of the patients 1000 times from the same specimen to verify the effectiveness of the model.

Results: We found that the percentage of eosinophils, a high–fluorescence-reticulocyte ratio, and creatinine had better discriminatory power than other factors. Age and underlying diseases were not significant for discrimination. The model correctly discriminated 77.1% of patients. In the final validation step, we observed that the model had an overall predictive rate of 81.3%.

Conclusion: We developed a laboratory model for COVID-19 pneumonia in patients with mild to moderate symptoms. In the clinical setting, the model will be able to predict and differentiate pneumonia vs nonpneumonia before any lung computed tomography findings. In addition, the percentage of eosinophils, a high–fluorescence-reticulocyte ratio, and creatinine were considered protective factors against lung infection in patients without pneumonia.

Keywords: COVID-19 infection, pneumonia, non-pneumonia, predictive model, protective factor, laboratory examination

Since the outbreak of COVID-19 in Wuhan, China, in December 2019, the COVID-19 epidemic has developed rapidly.[1] By April 2020, the epidemic had affected most countries and regions in the world.[2] The disease has caused serious global health and social problems.

Abbreviations:

CT, computed tomography; RT-PCR, reverse-transcription polymerase chain reaction; HDL, high-density lipoprotein.

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Patients with COVID-19 with severe symptoms usually die of pneumonia within a short period of time after infection, whereas a small proportion of patients die of other causes.[3] Mild acute respiratory infection symptoms, such as fever, dry cough, and fatigue, usually occur in the early stages of COVID-19,[4] but those who develop acute respiratory distress syndrome, acute respiratory failure, multiple organ failure, and other fatal complications die rapidly.[5] Generally, patients infected with COVID-19 without pneumonia recover, and asymptomatic infection is not life-threatening.[6] However, a specific treatment method for COVID-19 has not been fully developed.[7]

Because of decreased immunity and underlying diseases,[8] the symptoms and mortality associated with COVID-19 in older adults are more serious.[9] Older adult patients are more susceptible to viral infections and death[10] and have more underlying diseases, such as hypertension, hyperlipidemia, diabetes, and rheumatoid arthritis.[10-12] However, it is not clear whether age and underlying disease can predict pneumonia. Current research on COVID-19 has focused on

the epidemiology and clinical characteristics of patients, but information on the susceptibility to pneumonia has not been clear.

Pneumonia clearly plays a vital role in the prognosis of COVID-19. Therefore, we were committed to finding a way to identify whether a patient was susceptible to pneumonia before a chest computed tomography (CT) scan or before symptoms of pneumonia appear. Quickly identifying such patients will help prevent more serious cases of infection. It is a way to fight the death caused by COVID-19 infection. This research aimed to develop a model that can accurately distinguish patients with pneumonia from those without pneumonia in patients with COVID-19 and determine the factors that are significant in fighting infections in the lungs. The study investigated a group of patients at Hefei Second People's Hospital in China. Herein, we report our epidemiological, clinical, radiological, and laboratory examination results.

Methods

Study Design and Participants

We enrolled 50 patients who were diagnosed with COVID-19 at Hefei Second People's Hospital and Affiliated Hospital of Anhui Medical University. The inclusion criteria were as follows: patients with a confirmed diagnosis of COVID-19, patients with mild to moderate symptoms, men or women, patients with or without underlying diseases, and patients without any signs of death, including any symptoms of acute respiratory distress and/or failure of any organ. Patients with mild to moderate symptoms were defined by fever, fatigue, and smell and taste disorders, with or without respiratory symptoms (ie, coughing, sputum, and lung CT or X-ray showing pneumonia). They were managed in the hospital from January 2020 to April 2020, and as the final outcome all patients were discharged from the hospital. Patients were defined as having COVID-19 infection if they met any of the following criteria: (i) respiratory tract or blood specimens that were positive for SARS-CoV-2 per nucleic acid test using real-time fluorescent reverse-transcription polymerase chain reaction (RT-PCR) and/or (ii) through the SARS-CoV-2 gene sequencing method, a new virus found in the respiratory tract or blood specimens that was highly homologous to COVID-19.

These data were used to construct a predictive model of pneumonia. The research was approved by the Institutional Review Board of Anhui Medical University and complied with the Declaration of Helsinki. Patients were told verbally that their data would be used for medical research anonymously. After obtaining their permission, we collected written informed consent.

Variable Measurement

Previous medical history, age, and symptoms (fever, fatigue, smell and taste disorders, and respiratory symptoms) were recorded daily by resident physicians and nurses. The laboratory data after hospital admission were collected. Routine blood tests were performed multiple times over the course of the disease, and they were a part of the patient's standard care and testing. The blood test used 2 mL of blood from the cubital vein of the patient, and the blood was stored in an EDTA-dipotassium anticoagulation tube. Routine blood tests were completed using an automatic blood analyzer (Hitachi 7600 automatic analyser, Japan) and electrical impedance methods. The items obtained included the number of red blood cells, hemoglobin, white blood cells, platelet counts, absolute lymphocyte values, absolute intermediate cells, absolute neutrophils, lymphocyte percentage, intermediate cell percentage, percentage of neutral granulocytes, hematocrit, average red blood cell volume, average red blood cell hemoglobin, average hemoglobin concentration, red blood cell distribution width, average platelet volume, platelet distribution width, and platelet hematology.

For blood biochemistry indexes, 4 mL of venous blood was drawn, and the supernatant was removed and put into an automatic biochemical instrument (Hitachi, Japan) for analysis. Enzyme assay was used to obtain the reaction rate data for enzyme kinetic analysis, which was used to test the myocardial enzyme spectrum and adenosine dehydrogenase. Total bile acid was detected using an enzymatic cycle method. γ-glutamyltransferase was measured using the gamma-glutamyl-p-nitroanilide (GPNA) substrate method. Carbon dioxide was measured using a phosphoenolpyruvate carboxylase (PEPC) enzymatic method. Uric acid was measured using the oxidase method. Apolipoprotein A1 and apolipoprotein B were measured using the immunoturbidimetric method. Inorganic phosphorus was measured using the phosphomolybdate method.

Lab Medicine 2021;52;e104–e114 e105
DOI: 10.1093/labmed/lmab015

High-density lipoprotein (HDL) cholesterol and C-reactive protein were measured using the immunoturbidimetric method. Magnesium was measured using the xylene blue method. Creatinine was measured using the sarcosine oxidase method. Urea was measured using the urease-glutamate dehydrogenase method. Triglycerides were measured using the glycerol phosphate oxidase (GPO-PAP) method. Cystatin C was measured using a latex-enhanced immunoturbidimetric method. Total bile acid was measured using an enzyme cycle method. Total bilirubin was determined by using the vanadate oxidation method. Albumin was determined by using the bromocresol green method. Total protein was determined by using the biuret method. Hypersensitive C-reactive protein was determined by using immunofluorescence chromatography.

Lung CT Scan

Using the Siemens Somatom definition 64-row spiral CT scan, with patients in the supine position and the head at an incline, the researchers told patients to hold their breath during the scan. The scanning range was from the top to the bottom of both lungs and the cross-sectional area. The scanning parameters were as follows: tube voltage 120 kV, tube current 320 mA, matrix 512 × 512, layer thickness and layer moment 5 mm.[13]

Statistical Analysis

For data analyses, we selected the examination data taken at the most severe time in the course of the disease (that is, when the patient's self-reported symptoms, including fever, cough, chest pain, and/or muscle weakness, were the most severe).

The baseline demographic and clinical characteristics of all participants at the time of enrollment are presented as continuous variables and categorical variables (**Table 1**). The CT scan results divided all patients into 2 groups: patients with pneumonia and patients without pneumonia. The χ^2 test, 1-way analysis of variance, and Kruskal-Wallis tests were used to analyze the differences in these variables between the 2 groups. We used SPSS version 24 was used for statistical analyses, and G-power was used to determine whether each step in the statistics reached sufficient power.

In the initial factor selection step, we selected potential predictors by comparing the 2 groups, and the factors showing group differences were considered candidate predictors. In the second factor selection step, regularized regression with least absolute shrinkage and selection operator (LASSO) variable selection was used.[14] The LASSO penalization selected important predictors by shrinking the coefficients of weaker predictors to zero and excluded predictors with estimated zero coefficients from the final sparse prediction model. To avoid model overfitting in the training samples, the variable selection used 10-fold cross-validation[15] to select the best adjustment or penalty level, which was measured by the Bayesian information criterion.[16]

In the model development step, a stepwise logistic regression generalized an estimating equation, with the factors selected above entered as independent variables and the CT scan results entered as dependent variables. In the final validation step, a decision tree with the growth method of Chi-square automatic interaction detector (CHAID) and split sample tests were conducted, and random allocation of the whole sample (including all patients) was 50% for training and 50% for test sampling for 1000 times. The logistic model derived from the above was applied in the 50% randomly selected validation sample to calculate the predicted probabilities for each patient.

Hospitalization and Laboratory Tests

All patients were actively treated after admission, with daily droplet isolation, contact isolation, and routine care for Class A infectious diseases. The daily medication regimen of patients included lopinavir/ritonavir tablets 1000 mg (500 mg tablet × 2), 2 mL nebulized saline by inhalation, injection of 5 million units of recombinant human interferon alpha 2b, Chinese medicine decoction, vitamin C tablet 0.2 g, arbidol tablet 0.2 g, thymosin enteric-coated tablet 15 mg, and chloroquine hydrogen sulfate tablet 300 mg. Patient symptoms all improved after 4 to 34 days of treatment, and all patients were discharged home from the hospital.

The oropharyngeal swab nucleic acid test was assessed by the Centers for Disease Control and Prevention in Yaohai District, Hefei City. By using a fluorescent PCR method, a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used to extract RNA from the specimens from each patient into 50 μL of eluate. Forward (5′-CCT ACTAAATTAAATGATCTCTGCTTTACT-3′) and reverse (5′-CAAGCTATAACGCAGCCTGTA-3′) primers targeted the S gene of COVID-19 for determination of viral RNA. Real-time nucleic acid amplification tests were performed

Table 1: Baseline demographic and clinical characteristics of all participants

	Non-Pneumonia		Pneumonia			
	Mean or %	SD	Mean or %	SD	*t* or χ2	Sig.
Number	24	∅	26	∅		
Age (years)	40.25	18.32	50.42	14.18	-2.02	.05[a]
Hospital Stay (days)	20.83	19.50	17.32	10.59	0.60	.56
Nucleic acid test (times)	5.83	4.67	2.92	0.64	2.16	.05
Gender					0.10	.75
Male	50%	∅	55%	∅		
Female	50%	∅	45%	∅		
Underlying Disease					0.04	.85
Yes	42%	∅	45%	∅		
No	58%	∅	55%	∅		
Family cluster outbreak					3.29	.07
Yes	100%	∅	76%	∅		
No	0	∅	24%	∅		

SD, standard deviation; Sig., significance.
[a]*P < .05.*

using the QuantiNovaSYBR Green RT-PCR Kit (Qiagen) in a LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland). Reactions were incubated at 50°C for 10 minutes and 95°C for 2 minutes, followed by 45 cycles at 95°C for 5 seconds and 60°C for 30 seconds and then subjected to melting curve analysis (95°C for 5 seconds, 65°C for 1 minute, followed by a gradual increase in temperature to 97°C with continuous recording of fluorescence).

Respiratory specimens from the patients were collected separately and tested for influenza A and B viruses and respiratory syncytial virus using the Xpert Xpress Flu/RSV assay (GeneXpert System, Cepheid, Sunnyvale, CA). Specimens were tested with the BioFire FilmArray Respiratory Panel 2 Plus (bioMérieux, Marcy l'Etoile, France) to detect the presence of respiratory microbial pathogens, including coronavirus, adenovirus, respiratory syncytial virus, influenza A virus, influenza B virus, parainfluenza virus, *Mycoplasma pneumoniae, Chlamydia pneumoniae,* and *Legionella pneumophila.*

Routine urine analysis was completed using urine dip strips and a dry chemical method, along with a urine analyzer and colorimetry. The main parameters evaluated in the urine included urine color, urine pH, urine specific gravity, qualitative protein, and microscopic examination. Routine fecal examination was the direct microscopic examination of slides under a microscope. The main parameters analyzed in feces included stool color and hardness, mucus, and a microscopic examination of the stool for helminthiasis (hookworm eggs, roundworm eggs, and whipworm eggs) and other parasites.

Results

Among 50 patients with COVID-19 with mild to moderate symptoms, 26 had positive signs on lung CT leading to a COVID-19 pneumonia diagnosis, and 24 were diagnosed with COVID-19 infection without pneumonia. Twenty-three patients had underlying diseases before they entered the hospital, including hypertension (n = 10), hyperlipidemia (10), diabetes (5), rheumatoid arthritis (1), chronic bronchitis with emphysema (2), cervical spondylosis (1), femoral head necrosis (1), cerebral infarction (1), chronic hypothyroidism (1), fatty liver (1), and chronic superficial gastritis (2); in addition, 1 patient was pregnant and another was breastfeeding. The demographic characteristics of the two groups are shown in **Table 1**. Patients with pneumonia (ages 50.42 ± 14.18 years) were older than the patients without pneumonia (ages 40.25 ± 18.32 years) ($P < 0.05$). Otherwise, the 2 groups were similar regarding the time of hospital stay, sex composition, ratio of underlying disease, and ratio of family cluster outbreaks.

In the initial factor selection step, we found that patients without pneumonia had a greater number of lymphocytes

DOI: 10.1093/labmed/lmab015

Table 2: Group Differences on Blood Cell and Biochemistry Measures

	Non-Pneumonia		Pneumonia			
Blood Cells	**Mean**	**SD**	**Mean**	**SD**	***t***	**Sig.**
Lymphocytes	1.73	0.66	1.36	0.50	2.07	.04
Eosinophils	0.12	0.08	0.03	0.04	5.12	.00
Eosinophils%	2.09	0.83	0.62	0.52	5.12	.00
Basophils	0.40	0.26	0.31	0.18	2.10	.04
Reticulocyte absolute value	58.59	25.56	40.51	16.71	2.77	.01
Reticulocyte%	1.40	0.89	0.90	0.40	2.65	.01
High fluorescence reticulocyte ratio	1.34	0.91	0.15	0.41	2.40	.02
Biochemistry	**Mean**	**SD**	**Mean**	**SD**	***t***	**Sig.**
Calcium	2.32	0.09	2.20	0.15	2.46	.02
Creatinine	52.61	18.00	66.08	17.62	-2.22	.03
Urea/Creatinine	0.08	0.02	0.06	0.02	2.77	.01
Globulin	22.99	5.51	26.30	2.95	-2.63	.05
Albumin/Globulin	1.92	0.58	1.51	0.23	3.54	.00
Creatine kinase	46.00	14.03	77.11	15.92	-3.27	.00
Lactate dehydrogenase	178.91	47.05	251.24	76.92	-2.95	.01
HDL/CHOL	28.58	6.40	23.32	5.76	2.59	.01
Prealbumin	211.72	73.67	158.57	55.04	2.60	.01
Apolipoprotein A1	1.18	0.19	1.00	0.21	2.47	.02
SAA	45.46	72.78	110.63	69.04	-2.72	.01

HDL/CHOL, high-density lipoprotein/cholesterol; SAA, serum amyloid A; SD, standard deviation; Sig., significance.

and eosinophils, a higher percentage of eosinophils, a greater number of basophils, a greater absolute value of reticulocytes, a higher percentage of reticulocytes, and a higher ratio of high-fluorescent reticulocytes than patients with pneumonia, $P < .05$ (**Table 2**). G-power analyses determined that the effect size of these factors between the 2 groups ranged from 0.63 to 1.28, and the power of each comparison ranged from 0.49 to 0.97. Patients without pneumonia also had higher levels of calcium, lower creatinine, a higher urea/creatinine ratio, lower globulin, a higher albumin/globulin ratio, lower creatine kinase, lower lactate dehydrogenase, a higher HDL/cholesterol ratio, higher pre-albumin, higher apolipoprotein A1, and lower serum amyloid A (SAA) levels in blood than patients with pneumonia ($P < .05$; **Table 2**). G-power analyses determined that the effect size of these factors between the 2 groups ranged from 0.75 to 0.93, and the power of each comparison ranged from 0.60 to 0.75. In the second factor selection step, we observed that the percentage of eosinophils, the absolute value of reticulocytes, the high–fluorescence-reticulocyte ratio, creatinine, the albumin/globulin ratio, and lactate dehydrogenase survived the LASSO penalty and were selected as potential predictive factors (see **Tables 3** and **4**).

With a backward stepwise logistic regression model (see **Table 5**), age, the percentage of eosinophils, the absolute value of reticulocytes, the high–fluorescence-reticulocyte ratio, creatinine, the albumin/globulin ratio, and lactate dehydrogenase were entered as independent variables, and the pneumonia group was entered as the dependent variable. We found that the percentage of eosinophils, the high–fluorescence-reticulocyte ratio, and creatinine had better discriminatory power than the other factors. The predictive rate of the model was 77.1%, with $\chi^2 = 35.25$, Cox-Snell $R^2 = .52$, and $P < .05$.

In the final validation step, a decision tree with the growth method of CHAID and split sample (ie, all patients) tests were conducted. The logistic model derived from above was applied in the 50% randomly selected validation sample to calculate predicted probabilities for each patient for 1000 times. We observed that the validation samples had an overall predictive rate of 81.3%.

Table 3: Lasso coefficient (the factors left in rows 28-31 were selected as the potential predictors)

No.	Number of selected predictors	Number of lymphocytes	Eosinophils	Eosinophils%	Basophils	Reticulocytes absolute value	Reticulocytes percentage	High fluorescent reticulocytes ratio
1	7	0.322	-0.271	0.733	-0.334	0.164	0.089	0.353
2	7	0.301	-0.148	0.629	-0.301	0.165	0.043	0.341
3	7	0.281	-0.029	0.527	-0.268	0.16	0.004	0.329
4	6	0.263	0.037	0.47	-0.238	0.146	0	0.309
5	6	0.245	0.03	0.469	-0.207	0.138	0	0.289
6	6	0.225	0.024	0.47	-0.172	0.132	0	0.262
7	6	0.206	0.017	0.472	-0.138	0.126	0	0.236
8	5	0.149	0	0.488	0.022	0.125	0	0.153
9	5	0.142	0	0.482	0.013	0.12	0	0.146
10	7	0.136	0.001	0.473	0.003	0.11	0.006	0.141
11	4	0.127	0	0.471	0	0.111	0	0.133
12	5	0.12	0.002	0.461	0	0.104	0	0.129
13	4	0.111	0	0.46	0	0.101	0	0.119
14	4	0.104	0	0.454	0	0.096	0	0.112
15	4	0.096	0	0.449	0	0.092	0	0.105
16	4	0.088	0	0.443	0	0.087	0	0.098
17	4	0.08	0	0.438	0	0.082	0	0.091
18	4	0.074	0	0.43	0	0.075	0	0.088
19	4	0.065	0	0.427	0	0.072	0	0.077
20	4	0.058	0	0.421	0	0.066	0	0.072
21	4	0.05	0	0.416	0	0.063	0	0.063
22	4	0.042	0	0.411	0	0.058	0	0.056
23	4	0.034	0	0.405	0	0.053	0	0.049
24	4	0.027	0	0.4	0	0.047	0	0.043
25	4	0.019	0	0.395	0	0.044	0	0.035
26	4	0.011	0	0.389	0	0.039	0	0.028
27	4	0.004	0	0.384	0	0.034	0	0.021
28	3	0	0	0.378	0	0.028	0	0.014
29	3	0	0	0.37	0	0.022	0	0.009
30	3	0	0	0.363	0	0.016	0	0.003
31	3	0	0	0.354	0	0.008	0	0
32	2	0	0.002	0.345	0	0	0	0
33	1	0	0	0.336	0	0	0	0
34	1	0	0	0.326	0	0	0	0
35	1	0	0	0.316	0	0	0	0
36	1	0	0	0.306	0	0	0	0
37	1	0	0	0.296	0	0	0	0
38	1	0	0	0.286	0	0	0	0
39	1	0	0	0.276	0	0	0	0
40	1	0	0	0.266	0	0	0	0
41	1	0	0	0.256	0	0	0	0

Table 3. Continued

No.	Number of selected predictors	Number of lymphocytes	Eosinophils	Eosinophils%	Basophils	Reticulocytes absolute value	Reticulocytes percentage	High fluorescent reticulocytes ratio
42	1	0	0	0.246	0	0	0	0
43	1	0	0	0.236	0	0	0	0
44	1	0	0	0.226	0	0	0	0
45	1	0	0	0.216	0	0	0	0
46	1	0	0	0.206	0	0	0	0
47	1	0	0	0.196	0	0	0	0
48	1	0	0	0.186	0	0	0	0
49	1	0	0	0.176	0	0	0	0
50	1	0	0	0.166	0	0	0	0
51	1	0	0	0.156	0	0	0	0

Table 4: Lasso coefficient (the factors left in rows 39-45 were selected as the potential predictors)

No.	Number of selected predictor	Calcium	Creatinine	Urea/ creatinine	Globulin	Albumin/ globulin	Creatine kinase	Lactatede hydrogenase	HDL/CHOL	Prealbumin	Apolipoprotein A1	SAA
1	11	0.228	0.146	0.33	-0.507	0.125	-0.245	-0.181	0.434	-0.276	-0.2	-0.137
2	11	0.202	0.065	0.29	-0.453	0.124	-0.228	-0.206	0.36	-0.161	-0.155	0.032
3	11	0.158	-0.006	0.255	-0.405	0.121	-0.223	-0.194	0.332	-0.117	-0.145	0.005
4	10	0.136	-0.026	0.235	-0.372	0.124	-0.213	-0.181	0.312	-0.08	-0.127	0
5	10	0.113	-0.046	0.216	-0.346	0.122	-0.203	-0.175	0.29	-0.048	-0.107	0
6	10	0.091	-0.069	0.196	-0.317	0.121	-0.194	-0.169	0.267	-0.013	-0.086	0
7	8	0.086	0	0.215	-0.325	0.114	-0.2	-0.165	0.27	0	-0.093	0
8	8	0.079	0	0.201	-0.313	0.11	-0.189	-0.168	0.251	0	-0.072	0
9	8	0.073	0	0.187	-0.302	0.106	-0.179	-0.171	0.233	0	-0.052	0
10	8	0.069	0	0.174	-0.291	0.103	-0.168	-0.172	0.216	0	-0.032	0
11	8	0.047	-0.113	0.122	-0.253	0.102	-0.135	-0.175	0.182	0	0	0
12	8	0.041	-0.113	0.112	-0.242	0.104	-0.126	-0.174	0.173	0	0	0
13	8	0.035	-0.114	0.103	-0.231	0.107	-0.117	-0.172	0.164	0	0	0
14	8	0.03	-0.115	0.094	-0.22	0.109	-0.108	-0.17	0.155	0	0	0
15	8	0.025	-0.116	0.085	-0.21	0.112	-0.099	-0.167	0.147	0	0	0
16	8	0.021	-0.117	0.077	-0.199	0.115	-0.09	-0.164	0.139	0	0	0
17	8	0.017	-0.117	0.068	-0.19	0.117	-0.081	-0.16	0.131	0	0	0

Table 4: Continued

No.	Number of selected predictor	Calcium	Creatinine	Urea/ creatinine	Globulin	Albumin/ globulin	Creatine kinase	Lactatede hydrogenase	HDL/CHOL	Prealbumin	Apolipoprotein A1	SAA
18	8	0.014	-0.118	0.059	-0.181	0.118	-0.071	-0.157	0.123	0	0	0
19	8	0.011	-0.119	0.049	-0.173	0.118	-0.061	-0.155	0.114	0	0	0
20	8	0.008	-0.119	0.04	-0.164	0.118	-0.051	-0.152	0.106	0	0	0
21	8	0.005	-0.12	0.031	-0.156	0.119	-0.042	-0.149	0.098	0	0	0
22	8	0.002	-0.121	0.022	-0.147	0.119	-0.032	-0.146	0.091	0	0	0
23	7	0	-0.122	0.013	-0.139	0.12	-0.023	-0.142	0.083	0	0	0
24	7	0	-0.122	0.004	-0.131	0.12	-0.013	-0.139	0.075	0	0	0
25	6	0	-0.121	0	-0.123	0.121	-0.004	-0.135	0.067	0	0	0
26	5	0	-0.116	0	-0.116	0.121	0	-0.129	0.059	0	0	0
27	5	0	-0.111	0	-0.107	0.124	0	-0.123	0.051	0	0	0
28	5	0	-0.106	0	-0.098	0.127	0	-0.116	0.044	0	0	0
29	5	0	-0.1	0	-0.09	0.129	0	-0.109	0.036	0	0	0
30	5	0	-0.095	0	-0.082	0.131	0	-0.102	0.029	0	0	0
31	5	0	-0.09	0	-0.074	0.133	0	-0.096	0.022	0	0	0
32	5	0	-0.085	0	-0.067	0.134	0	-0.089	0.015	0	0	0
33	5	0	-0.08	0	-0.057	0.138	0	-0.082	0.007	0	0	0
34	5	0	-0.075	0	-0.051	0.138	0	-0.076	0	0	0	0
35	4	0	-0.069	0	-0.026	0.161	0	-0.064	0	0	0	0
36	4	0	-0.062	0	-0.017	0.162	0	-0.057	0	0	0	0
37	4	0	-0.056	0	-0.009	0.164	0	-0.05	0	0	0	0
38	4	0	-0.049	0	-0.001	0.166	0	-0.043	0	0	0	0
39	3	0	-0.042	0	0	0.162	0	-0.036	0	0	0	0
40	3	0	-0.035	0	0	0.156	0	-0.03	0	0	0	0
41	3	0	-0.028	0	0	0.15	0	-0.024	0	0	0	0
42	3	0	-0.021	0	0	0.145	0	-0.018	0	0	0	0
43	3	0	-0.014	0	0	0.139	0	-0.012	0	0	0	0
44	3	0	-0.007	0	0	0.134	0	-0.006	0	0	0	0
45	3	0	0	0	0	0.128	0	0	0	0	0	0
46	1	0	0	0	0	0.118	0	0	0	0	0	0
47	1	0	0	0	0	0.108	0	0	0	0	0	0
48	1	0	0	0	0	0.098	0	0	0	0	0	0
49	1	0	0	0	0	0.088	0	0	0	0	0	0
50	1	0	0	0	0	0.078	0	0	0	0	0	0
51	0	0	0	0	0	0	0	0	0	0	0	0

Table 5: The Stepwise Regression Model

	B	S.E.	Wald	DoF	Sig.	Exp (B)	CI of EXP(B)	
							Lower limit	Upper limit
Eosinophil%	5.154	1.839	7.853	1	.005	173.047	4.707	6361.383
High fluorescent reticulocyte ratio	1.039	0.583	3.174	1	.075	2.827	0.901	8.865
Creatinine	-0.124	0.06	4.297	1	.038	0.883	0.785	0.993
Constant	-0.764	2.938	0.068	1	.795	0.466		

B, coefficients; S.E., standard error; Wald, Wald test; DoF, degree of freedom; Sig., significance; Exp (B), exponentiation of the B coefficient; CI, confidence interval.

Discussion

The study included 50 patients with mild to moderate symptoms of COVID-19. We have established a laboratory model that can predict pneumonia through readily available laboratory measures. The model showed good discrimination, and external verification was satisfactory. This is the first study to determine the important factors that may fight COVID-19 pneumonia or potential protective factors in patients with mild to moderate symptoms.

Generally, age and underlying disease are regarded as risk factors for COVID-19 pneumonia. Older adults are generally at higher risk of chronic diseases and are more susceptible to infection.[17] Age is a risk factor for poor prognosis in patients with COVID-19, partly because age-related immune dysfunction is caused by low-grade chronic inflammation.[18] In addition, older adult patients may also have other risk factors, such as comorbidities and sarcopenia.[19,20] For example, a history of hypertension is an important risk indicator of the MuLBSTA score, a viral pneumonia death warning model.[21] Hypertension has been found to be a predictor of death in patients with COVID-19.[18] However, in this study, neither age nor underlying disease was an important factor in distinguishing the pneumonia group. Although the age of patients with pneumonia was relatively higher than for those without pneumonia, this finding may have been because our patients had mild to moderate symptoms rather than severe symptoms, different from previous studies.

With the predictive model, we found that the percentage of eosinophils, the high–fluorescence-reticulocyte ratio, and creatinine in the blood were good predictors or discriminators for patients with and without pneumonia. Patients without pneumonia had a higher percentage of eosinophils, a greater high–fluorescence-reticulocyte ratio, and lower creatinine levels than patients with pneumonia.

Eosinophils are produced by bone marrow stem cells and account for 1% to 5% of the total number of white blood cells in the blood.[22] Eosinophils play an important role in adaptive immune function, specifically resisting viruses.[23] As immune modulators, eosinophils are not only associated with the effector arm of adaptive immunity but also trigger a polarized adaptive response process.[24] In diseases caused by respiratory viruses, the blood and immune organs are severely damaged[25] and viruses directly inhibit the proliferation of bone marrow cells. Some clinical studies have found that viral infections cause a decrease in the percentage of eosinophils in routine blood tests.[26] The relatively higher percentage of eosinophils in our patients without pneumonia may indicate better adaptive immunity and a better polarized adaptive response process, which helped these patients avoid lung infection.

Studies have shown that a variety of viral infections cause bone marrow hematopoietic arrest and inhibit bone marrow cell proliferation.[27–29] After the virus invades, it binds to red blood cell membrane proteins and damages red blood cell production, resulting in a decrease in the number of red blood cell lines and reticulocytes in the blood.[30] The number of reticulocytes has been used to clinically judge the severity of viral infections and the hematopoietic function of bone marrow.[31] Viral infection in this study was associated with decreased reticulocytes, and the relatively higher level of the high–fluorescence-reticulocyte ratio indicated better bone marrow cell proliferation, which was also a factor that helped patients without pneumonia avoid lung infection.

The abnormal levels of urea, creatinine, and the urea/creatinine ratio were clinically indicative of impaired renal

function.[32,33] In addition to damaging the respiratory system, COVID-19 has also been found to harm the kidneys and liver.[34] One study showed that approximately 3% to 10% of patients with COVID-19 had abnormal renal function, including a significant increase in creatinine and/or blood urea nitrogen.[35] In this study, patients without pneumonia had a lower level of creatinine, indicating a better kidney function.

Conclusion

The laboratory model showed good discriminatory power with a predictive rate of 77.1%, a sensitivity of 100.00%, and a negative predictive value of 100.00%. The validation samples (ie, patients) had an overall predictive rate of 81.3%. Among the patients with mild to moderate symptoms of COVID-19, age and underlying diseases were not significant in the discrimination between the pneumonia and nonpneumonia groups. The percentage of eosinophils, the high–fluorescence-reticulocyte ratio, and creatinine in the blood were good discriminators between the 2 groups. Patients without pneumonia had a higher percentage of eosinophils, a greater high–fluorescence-reticulocyte ratio, and lower creatinine levels than patients with pneumonia. The relatively higher percentage of eosinophils may indicate better adaptive immunity and a better polarized adaptive response process. The relatively higher level of the high–fluorescence-reticulocyte ratio indicated better bone marrow cell proliferation, and the lower level of creatinine may have indicated better kidney function. These factors may be protective factors for patients without pneumonia against lung infection. **LM**

Acknowledgments

We thank Anhui Medical University for giving ethical approval. We are also grateful for the laboratory personnel working in all targeted laboratories for giving permission and participation in the data collection activities. The original data for this study are available from the corresponding authors upon request. The study was ethically approved by the Institutional Review Committee of Anhui Medical University, and official permission was obtained from all participating health facilities. Data from each facility were presented anonymously using code. All discordant results were also communicated to the respective laboratories.

Funding

The study was supported by the fund of Anhui Province Key Research and Development Plan Project (1804h08020233).

Conflict of Interest

The authors report no biomedical financial interests or potential conflicts of interest.

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DOI: 10.1093/labmed/lmab015

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Special Report

Initial Clinical Laboratory Response to COVID-19: A Survey of Medical Laboratory Professionals

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Laboratory Medicine 2021;52:e115-e124

DOI: 10.1093/labmed/lmab021

ABSTRACT

Objective: To explore the experiences of medical laboratory professionals (MLPs) and their perceptions of the needs of clinical laboratories in response to COVID-19.

Methods: We surveyed laboratory professionals working in United States clinical laboratories during the initial months of the pandemic.

Results: Overall clinical laboratory testing and overtime work for laboratorians decreased during the first months of the pandemic. Laboratory professionals reported better or unchanged job satisfaction, feelings toward their work, and morale in their workplace, which were related to healthcare facility and laboratory leadership response. They reported receiving in-kind gifts, but no hazard pay, for their essential work. Important supply needs included reagents and personal protective equipment (PPE).

Conclusion: The response by healthcare facilities and laboratory leadership can influence MLPs job satisfaction, feelings toward their work, and laboratory morale during a pandemic. Current COVID-19 laboratory testing management, in the absence of sufficient reagents and supplies, cannot fully address the needs of clinical laboratories.

Keywords: COVID-19, laboratory personnel, health workforce, clinical laboratory services, management/administration, occupational safety

The transmission of the novel coronavirus SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), which is the causative agent of the coronavirus disease 2019 (COVID-19), is a worrisome possibility in health care settings.[1] Because of the risk for health care workers becoming ill or having to take time off to provide care to sick relatives, the Centers for Disease Control and Prevention (CDC) issued strategies to reduce potential staffing shortages of health care personnel (HCP), such as identifying additional personnel to work in case of shortages and establishing testing and tracking protocols.[2] During this ongoing crisis, the concerns of HCPs for their health and safety are heightened by the lack of personal protective equipment (PPE) and limited laboratory-testing resources.[3] Although testing is required to secure a diagnosis, increased demand and shortages of basic necessities, such as test kits, reagents, and supplies, were some of the main concerns during the first months of the pandemic.[3,4]

Abbreviations:

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; COVID-19, coronavirus disease 2019; CDC, Centers for Disease Control and Prevention; HCP, health care personnel; PPE, personal protective equipment; MLPs, medical laboratory professionals; US, United States; ASCP, American Society for Clinical Pathology; CAP, College of American Pathologists; ASCLS, American Society for Clinical Laboratory Science; CLS/MLS/MT, Clinical Laboratory Scientist/Medical Laboratory Scientist/Medical Technician; HEROES, Health and Economic Recovery Omnibus Emergency Solutions

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At the intersection between adequate diagnostic testing and HCP shortages are medical laboratory professionals (MLPs). They are key actors within the health care team who provide essential testing of biomarkers that aid in the detection, diagnosis, and treatment of diseases, as well as facilitating efforts to monitor health and engage in disease prevention.[5] Staffing shortages of MLPs were severe before the COVID-19 pandemic.[6] The vacancy rates in United States (US) medical laboratories rose from 7.2% in 2016 to 8.6% in 2018, as reported by the American Society for Clinical Pathology (ASCP) Vacancy Survey. Also, hiring of qualified laboratory professionals and rates of burnout among individuals working in the clinical laboratory were the top concerns for staffing of laboratories.[7–9]

Shortages of clinical laboratory resources and trained staff members can have a critical impact in providing a sufficient response during a pandemic.[10,11] Previously, the College of American Pathologists (CAP) reported the clinical-laboratory response to the H1N1 pandemic by surveying at the laboratory level. Also, the Medical Laboratory Observer annual survey and ASCP satisfaction and burnout survey have documented individual satisfaction and perception of staff shortages among MLPs.[11–13] However, at present, little is known about the practice conditions of HCP on the front lines, particularly the individual perceptions and needs of MLPs associated with the COVID-19 response. We designed and implemented a survey to explore the self-reported practices and experiences of MLPs, and to document their perceptions of the needs of clinical laboratories at the start of the pandemic.

Materials and Methods

This was a cross-sectional, anonymous, web-based survey of MLPs between April 29, 2020, and May 31, 2020. To recruit participants, we posted invitation messages in online forums and social media, and shared them with professional contacts and groups in which laboratory professionals receive communications. Our main source of recruitment was the membership of the American Society for Clinical Laboratory Science (ASCLS), which had more than 6,800 active members at the time of the study.[14] Only the data for individuals who provided informed consent was included in the sample. The inclusion criteria for the survey were that participants self-identify as a current employee of a clinical laboratory whose usual employment involved participating in the diagnostic laboratory process performing and providing results of clinical laboratory tests using human specimens. The instrument gathered demographic data about participants and their workplace using 32 closed-ended questions, with 8 questions containing branching that allowed for optional open-ended responses. Quantitative responses and demographic information collected were summarized with basic descriptive statistics, and statistical analyses were performed using STATA software, version 16.1.[15] Qualitative responses have been analyzed and will be presented separately. Individuals who completed the survey could opt in to participate in a drawing for the chance to win one of four $50 gift cards. This project was approved by the institutional review board at the University of Kansas Medical Center.

Results

There were 233 returned surveys, 178 (76.4%) of which were from participants who met inclusion criteria and completed the main instrument.[16] Average survey time was 17.83 minutes (minimum = 4.00; maximum = 229.00; SD = 25.6) excluding one participant who completed the survey during a period of multiple days (1219 minutes).

Demographic data collection on the 178 respondents varied with some answering one or few, but not all, questions. Survey participants were predominantly female and white, and their geographic location was heterogeneously spread across the main regions of the contiguous United States, with 39 states represented (Supplemental Tables S1and Table S2). The mean age of respondents was 42.9 years (n = 177; minimum = 21.0; maximum = 73.0), and average time in practice was 16.3 years (minimum <1.0; maximum = 47.0; Supplemental Table S3).

When asked about their current position, respondents reported a mean time in their current role of 6.4 years (minimum <1.0; maximum = 39.0) working, on average, 8.6 hours per shift (minimum = 4.0; maximum = 13.0); most of them worked the day shift (Supplemental Table S4). Respondents self-reported their position title, which yielded 105 distinct entries. We grouped these titles into categories using terminology from the US Bureau of Labor Statistics Occupational Outlook Handbook and other resources.[17–20] Most respondents were classified as CLS/MLS/MT (Clinical Laboratory Scientist/Medical Laboratory Scientist/Medical Technician), and more than one-third of respondents indicated the laboratory role/area associated with their position was generalist/core (Supplemental Table S5 and Table S6).

Most respondents reported bachelor's degree as their highest level of education (65%), and more than 78% of them also indicated at least 1 type of national credential, with most holding MLS(ASCP) certification (Supplemental Table S7 and Table S8). We received 139 responses from MLPs who reported membership with ASCLS, and some of them concurrently or solely held memberships from other professional laboratory societies (Supplemental

Table S9). The proportion of respondents working in a hospital clinical laboratory was 93%, with 33% of them working at hospitals with more than 500 beds (Supplemental Table S10).

Changes in Workload

Survey results showed a statistically significant difference between overtime work before and during the first months of the pandemic $\chi 2$ (4, N = 178) = 35.55, P <.001; All chi square values are given in the format: $\chi 2$ (degress of freedom, N = sample size) = chi-square statistic value, P = P value. Overall, 73.0% of MLPs reported working overtime at least once a month before the declaration of the COVID-19 pandemic by the CDC, with most reporting working overtime between 1 and 3 times a month (**Table 1**). After the pandemic was declared, overall reports of overtime work dropped to 57.9%. We were intrigued to discover that the proportion of respondents who said they worked overtime almost every day before the pandemic was 3.4% but during the first months of the pandemic, daily overtime work increased to 13.5%. On further analysis, there was statistically significant evidence (P <.05) of differences in overtime worked before and during the pandemic by area of the laboratory $\chi 2$ (6, n = 27) = 14.46, P = .03, hospital size $\chi 2$ (3, n = 30) = 10.89, P = .01, shift worked $\chi 2$ (3, n = 88) = 8.58, P = .03, and education level $\chi 2$ (3, n = 85) = 8.18, P = .04 (Supplemental Table S11).

Changes in workload were reported during the COVID-19 response, with 71.4% of respondents indicating that workload in the laboratory had decreased, 5.6% reporting no change, and 23.0% reporting that workload had increased (Supplemental Table S12). We noted that there was a statistically significant relationship between reports of decreased overtime work by MLPs and reports of a drop in workload early during the pandemic $\chi 2$ (2, n = 178) = 21.55, P <.001.

Table 1. Overtime Work Before and During the COVID-19 Pandemic

Overtime	No. (%)[a]	
	Before Pandemic	During Pandemic
Reports overtime work		
Between 1 and 3 times a month	72 (40.4%)	43 (24.2%)
Once a week	22 (12.4%)	9 (5.1%)
Between 2 and 4 times a week	30 (16.9%)	27 (15.2%)
Almost every day	6 (3.4%)	24 (13.5%)
Any overtime	130 (73.0%)	103 (57.9%)
No overtime	48 (27.0%)	75 (42.1%)
Total	**178 (100%)**	**178 (100%)**

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

Satisfaction, Morale, and Incentives

During the first months of the pandemic, 49.0% of MLPs reported improved satisfaction, with 54.0% reporting feeling better about their job, and 39.0% saying the morale was good in the laboratory. The relationship between reported positive satisfaction and perceived good morale in the workplace was statistically significant (t = –5.8610; P <.001). **Figure 1** shows the ratings of questions asking about satisfaction, feelings about work, morale, and response by their facility and laboratory leadership to the health emergency.

Perceptions of how well hospitals and laboratory leadership responded to the COVID-19 pandemic were related to participant ratings of job satisfaction, feelings toward work, and morale in their laboratory (**Table 2**). When participants rated their job satisfaction as better than before the pandemic, 86.0% of them also rated the response to the COVID-19 crisis by their hospital or health care facility as good. For those workers who said job satisfaction was worse, only 61.7% rated the response by their hospital as good. The relationships between job satisfaction and facility response were significant $\chi 2$ (2, n = 133) = 13.19, P = .001. Similarly, MLPs who rated job satisfaction as better than before considered the response by the laboratory leadership as good 82.6% of the time, but only 55.3% of MLPs who rated job satisfaction as worse said the response by their leadership was good $\chi 2$ (2, n = 133) = 11.64, P = .003. With respect to MLPs reporting better feelings toward their work, 82.3% said the response by the health care facility was good, but when workers said feelings toward their work were worse, only 58.3% rated the response by their facility as good $\chi 2$ (2, n = 132) = 10.55, P = .005.

Further, when participants reported the morale in the laboratory where they worked as being good, 88.4% said the response to the COVID-19 crisis by their health care facility was good and, among workers who said morale in their laboratory was poor, only 67.2% rated the response

DOI: 10.1093/labmed/lmab021

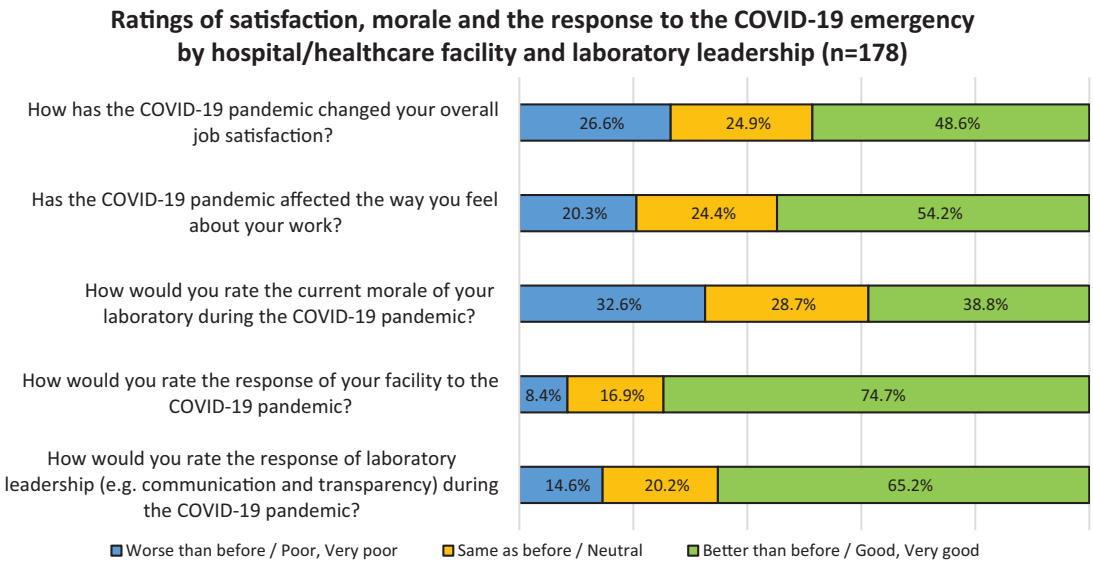

Figure 1

Ratings by participants of job satisfaction, feelings about work, workplace morale, and response by facility and laboratory leadership where they work during COVID-19.

Table 2. Laboratory Professionals Ratings of Hospital/Health Care Facility and Laboratory Leadership Response[a]

Facility Response	Job Satisfaction[b]			Feelings Toward Work[c]			Workplace Morale[d]		
	Worse	Better	Total	Worse	Better	Total	Poor	Good	Total
Poor	10 (21.3%)	3 (3.5%)	13 (100%)	8 (22.2%)	5 (5.2%)	13 (100%)	10 (17.2%)	2 (2.9%)	12 (100%)
Neutral	8 (17.0%)	9 (10.5%)	17 (100%)	7 (19.4%)	12 (12.5%)	19 (100%)	9 (15.5%)	6 (8.7%)	15 (100%)
Good	29 (61.7%)	74 (86.0%)	103 (100%)	21 (58.3%)	79 (82.3%)	100 (100%)	39 (67.2%)	61 (88.4%)	100 (100%)
Total	**47 (100%)**	**86 (100%)**	**133 (100%)**	**36 (100%)**	**96 (100%)**	**132 (100%)**	**58 (100%)**	**69 (100%)**	**127 (100%)**
Leadership Response									
Poor	10 (21.3%)	6 (7.0%)	16 (100%)	7 (19.4%)	12 (12.5%)	19 (100%)	14 (24.1%)	3 (4.4%)	17 (100%)
Neutral	11 (23.4%)	9 (10.5%)	20 (100%)	8 (22.2%)	11 (11.5%)	19 (100%)	20 (34.5%)	6 (8.7%)	26 (100%)
Good	26 (55.3%)	71 (82.6%)	97 (100%	21 (58.3%)	73 (76.0%)	94 (100%)	24 (41.4%)	60 (87.0%)	84 (100%)
Total	**47 (100%)**	**86 (100%)**	**133 (100%)**	**36 (100%)**	**96 (100%)**	**132 (100%)**	**58 (100%)**	**69 (100%)**	**127 (100%)**

[a] *Percentages may not total 100 because of rounding.*
[b] *n = 133.*
[c] *n = 132.*
[d] *n = 127.*

by their hospital as being good $\chi 2$ (2, n = 127) = 9.89, P = .007. Likewise, when respondents rated workplace morale as being good, they considered the response by the laboratory leadership as being good 87.0% of the time. In contrast, MLPs who rated the morale in their laboratory as being poor also rated the response by laboratory leadership as being only 41.4% of the time $\chi 2$ (2, n = 127) = 29.35, P <.001.

Also, we captured information associated with incentives received during the COVID-19 response. The proportion of participants who said they had received pay increases or hazard pay for work during the early months of the COVID-19 response was 5.1%. However, 82.0% of participants indicated they received in-kind incentives in appreciation of their work. A total of 64.0% said they received free meals and 50.0% reported

receiving thank you emails or cards at their workplace (Supplemental Table S13).

Impact on Workplace and Laboratory Needs

When asked if there had been changes in staffing conditions at their laboratory, 35.9% responded affirmatively, including 64 reports of staff being reassigned to other areas and 11 instances of staff being hired temporarily (Supplemental Table S14). When asked if cost-reduction measures (eg, reduction in work hours, being furloughed, or being laid off) had been applied at their workplace, 61.2% of participants reported such changes. Bivariate analysis assuming equal variances showed that reports of cost-reducing measures were higher for individuals who reported a poor response to COVID-19 by their hospital or health care facility (t = 2.9308; P = .004).

Most of the MLPs surveyed reported they were not worried about contracting the COVID-19 virus at their workplace (59.5%). Two-thirds of respondents said their laboratory allowed for proper social distancing per CDC guidelines, and 94.9% of them said that their facility had policies regarding face covering (Supplemental Table S15).

Regarding laboratory-testing capacity and methodologies, 36.0% of laboratory professionals reported that existing methods were revalidated, and 36.5% reported that new instruments were acquired for COVID-19 testing (Supplemental Table S16). **Table 3** shows the variety of testing options reported by participants. Respondents conveyed that 75.3% of test results for COVID-19 were reported out of the laboratory to clinicians and patients within 2 days, with 11.2% reporting test results in 3 to 4 days and 1.7% reporting test results in 5 days or more (Supplemental Table S16).

When asked whether there were any restrictions placed on COVID-19 testing at their facility, 61.2% of participants stated there were. **Table 4** shows the types of testing restrictions that were reported by respondents. Finally, when asked about necessary laboratory equipment that they considered to be in short supply or causing problems when trying to perform COVID-19 testing, most respondents said that PPE and reagents for testing were the most pressing needs for the laboratory. **Table 5** shows the breakdown of supply needs.

Multivariate logistic regressions (with robustness estimator) were calculated for worse satisfaction, worse feelings toward work, and worse morale during the pandemic, using incentives, ability to socially distance, facility and laboratory leadership response, the provision of COVID-19 testing in-house, and overtime work before and during the COVID-19 pandemic (Supplemental table S17). The results of these analyses indicated that, for MLPs who rated the response by facility leadership as being poor, the odds of reporting worse satisfaction were 8 times higher than for those who said the facility leadership response was not poor (OR, 8.647; 95% CI, 2.127–35.146). Similarly, for MLPs indicating poor response by facility leadership, the odds of reporting worse feelings toward work were 13.6 times higher (13.617; 3.368–55.062), and the odds of reporting worse morale in the laboratory were almost 4 times higher (3.96; 1.224–12.813), compared with those indicating that the response was not poor. Moreover, for MLPs that worked overtime before the COVID-19 pandemic, the odds of reporting worse morale in their laboratory were 6 times higher (6.008; 1.518–23.778) than for MLPs who did not report working

Table 3. Types of COVID-19 Tests Available

Response to Survey Question "What testing methodologies are available at your facility for COVID-19 testing? (Check all that apply)"	No. (%) of Responses[a])
In house testing using molecular amplification by RT-PCR	105 (32.9%)
In house testing using rapid antigen by nucleic acid testing	33 (10.4%)
In house testing using serology by ELISA	18 (5.6%)
In house testing using serology by rapid immunochromatography	13 (4.1%)
Specimen collection for molecular methods, tests sent out to reference laboratory	80 (25.1%)
Specimen collection for all methods, tests sent out to reference laboratory	46 (14.4%)
No specimen collection or testing for COVID-19 at all in this facility	2 (.6%)
Unsure/do not know/does not report	22 (7.0%)
Total	**319 (100%)**

COVID-19, coronavirus disease 2019.
[a]*Percentages may not total 100 because of rounding.*
[b]*n = 178.*

Lab Medicine 2021;52;e115–e124 e119
DOI: 10.1093/labmed/lmab021

Table 4. Restrictions to COVID-19 Testing by Patient Status[a]

Response to Survey Question "From the following list, which options describe restrictions to COVID-19 testing at your facility? (Please mark all that apply)"	No.(%) of Responses[b]
Inpatient	
No restrictions applied to COVID-19 testing	33 (10.0%)
Only patients who met CDC's COVID-19 risk factors are tested[a]	56 (16.9%)
Unsure/do not know/does not report	12 (3.6%)
Outpatient	
No restrictions applied to COVID-19 testing	5 (1.5%)
Only patients who met CDC's COVID-19 risk factors tested[a]	68 (20.5%)
Only patients being admitted with COVID-19 symptoms tested[a]	27 (8.2%)
Unsure/do not know/does not report	9 (2.7%)
Health Care Professionals	
No restrictions applied to COVID-19 testing	11 (3.3%)
Only staff who were in contact with people who tested positive	31 (9.4%)
Only staff who showed COVID-19 symptoms/fulfilled risk factors[a]	60 (18.1%)
Unsure/do not know/does not report	19 (5.7%)
Total reported restrictions	**331 (100%)**

COVID-19, coronavirus disease 2019; CDC, Centers for Disease Control and Prevention.
[a]*n = 178.*
[b]*COVID-19 symptoms and risk factors according to CDC guidelines.*

Table 5. Shortages of COVID-19 Testing Supplies and Equipment

Response to Survey Question "Which of the following resources would you say are in short supply or cause problems when trying to perform COVID-19 testing? (Please mark all that apply)"	No. (%) of Responses[a]
Instrumentation capacity	55 (16.6%)
PPE inventory	73 (22.0%)
Reagents for quality control	31 (9.4%)
Reagents for testing	90 (27.2%)
Other	42 (12.7%)
Unsure/do not know/does not report	40 (12.1%)
Total reported supply shortages	**331 (100%)**

COVID-19, coronavirus disease 2019; PPE, personal protective equipment.
[a]*n = 178.*

overtime before the pandemic. In contrast, having received a gift/recognition at work reduced the odds of reporting worse feelings toward work by 68.5% (0.315; 0.114–0.867), and performing tests for COVID-19 in-house reduced the odds of reporting worse morale in the laboratory by 55.4% (0.446; 0.175–1.134).

Discussion

This survey study captures the firsthand experiences of MLPs before and during the initial response to the COVID-19 pandemic. It documents the emotions of MLPs toward their work, their perceptions on how well their workplace has adapted to the pandemic, and their knowledge of the supply needs of clinical laboratories as they respond to COVID-19.

Despite a 20.8% reduction in reported overall overtime work, 57.9% of MLPs were working overtime during the first months of the pandemic, which provides evidence that shortages in the workforce continue to be a concern, as reported in previous years.[8] The immediate impacts of COVID-19 to laboratory workload were a reduction of overall testing and a general reduction in staff overtime.

One study looking at diabetes management during COVID-19 indicated that approximately 65% of clinicians

in the United States were worried about keeping their practices open due to low volume of reimbursable work, with visits for chronic care of asymptomatic patients down by 50%.[21] Fewer routine and preventive health services and cancellations of elective procedures may explain why certain areas of the laboratory experienced decreased volumes of testing. We were intrigued to find that the proportion of MLPs reporting overtime work daily after the pandemic declaration increased from 3.4% to 13.5%. This finding may have been related to overtime work for individuals involved in managing COVID-19 testing. The detection of the SARS-CoV-2 virus depends heavily on molecular diagnostics. These specialty tests require qualified professionals to implement tests and process specimens using sophisticated equipment and complex diagnostic instrumentation.[22] The other areas of the laboratory that reported an increase of everyday overtime included core/generalist, microbiology, and administration. The lattermost is particularly responsible for selection, validation, and preparation of test guides and documentation when a new viral test, such as the one for SARS-CoV-2, becomes part of the laboratory testing menu, which may have also impacted the increase reports of daily overtime work.[23,24]

MLPs reported increased or unchanged satisfaction with their job, rating the morale in their workplace as being better or unchanged. However, earlier literature[12] reports that 88% of MLPs are somewhat or very satisfied with their job, which suggests that reported job dissatisfaction may have doubled during the first months of the pandemic. Job satisfaction, feelings toward work, and perceived morale in the workplace were affected by the response to the pandemic by the administration particularly at the health care facility level. Further, those who were already working overtime before the pandemic were more likely to report that the morale in their laboratory worsened after the pandemic was declared.

Working in a positive laboratory environment can help employees feel efficient and can satisfy their need for professional fulfillment. Some management strategies that can be helpful during high-workload times include increased communication (constantly updating laboratory workers on the situation as it unfolds), keeping an open dialogue, and allowing input from MLPs during organizational decision-making.[25]

The Maslow Hierarchy of Needs indicates that a sense of professional accomplishment can be attained when workers receive recognition for their achievements, and that feelings toward work can also be influenced by perceptions of value and adequate compensation for the work performed.[26] Although the Centers for Medicare and Medicaid Services reportedly doubled the reimbursement for COVID-19 testing, this increase in reimbursement did not seem to provide monetary compensation for MLPs.[24] As testing for COVID-19 increased exponentially through the summer of 2020, the House of Representatives passed H.R. 6800, the Health and Economic Recovery Omnibus Emergency Solutions (HEROES) Act, which would help employers provide premium pay for essential workers.[27] The Heroes Act in its original form did not make it out of committee in the Senate, and was reintroduced as H.R. 8406 in September of 2020 followed by a companion senate bill (S. 4800), but neither bill received a vote in the 116th Congress.[28] Despite the lack of additional pay, many MLPs reported receiving recognition through in-kind gifts and messages of support for their work. We found that these gifts contributed to MLPs reporting positive feelings about their work.

The changes to laboratory staff in response to the COVID-19 pandemic were considerable, with many respondents reporting cost-reducing measures being implemented; however, others reported reassignments and temporary hiring of laboratory personnel. During the H1N1 epidemic, 16% of clinical laboratories surveyed by CAP reported staffing shortages, and approximately 6% indicated they had increased staffing to address testing needs; however, almost 90% of laboratories reported that the surge in testing did not impact patient care.[11] It is too early to know whether the COVID-19 pandemic will have a similar or different effect on clinical laboratories.

The use of universal precautions and strategies to mitigate transmission of the virus among HCPs is the first line of defense for essential workers.[2] Policy changes to establish protections against coronavirus transmission within the laboratory, as reported by participants, seemed to follow CDC guidelines regarding recommendations for use of a face covering.[2] However, according to our survey findings, more than one-third of respondents were unable to maintain the recommended physical separation while at work. Laboratories are usually closed spaces with no windows, which decreases opportunities for spatial distancing and for adequate ventilation. Even with new policies and procedures in place, 40.5% of MLPs were concerned that they may become exposed to COVID-19 through workplace transmission.

DOI: 10.1093/labmed/lmab021

We discovered that performing COVID-19 testing in-house, whether by incorporating new methodologies or revalidating existing methods, contributed to reports of better morale in clinical laboratories. MLPs have demonstrated a capacity to adapt by readily incorporating tests for an emerging disease to their repertoire and drastically modifying their work output to produce millions of test results for COVID-19 daily during a global crisis.[29] We find that test results for detection of SARS-CoV-2 were reportedly produced quickly, matching reports of increased testing despite the lack of adequate supplies and regardless of any guarantee that testing would be appropriately funded.[24,29,30]

As evidenced by our data, what worried MLPs the most was the availability of essential supplies to allow them to continue producing needed test results. Another factor to consider was the implementation of restrictions on SARS-CoV-2 tests, including testing only people who displayed symptoms and those who had come in close contact with someone with confirmed infection, among others.[31] In response to the H1N1 influenza pandemic of 2009, the CDC issued restrictive guidelines after experiencing a 100% surge over the usual influenza testing.[11] Our findings, which show that most testing was reserved for patients or health care professionals who had certain risk factors at the start of the pandemic, indicate that early implementation of testing restrictions may have helped manage the scaling up of COVID-19 testing. Notwithstanding these restrictions, the results of a survey of CLIA-certified laboratories found that scarcity of supplies for all types of testing continues to persist, particularly of commercial testing kits and consumables.[32]

This was an observational, cross-sectional study and thus possesses limitations inherent to its design. Due to the use of purposive sampling through professional organizations, our findings are not generalizable. People who belong to professional societies are already motivated to volunteer their time on behalf of the profession and participate in surveys, and their opinions may not represent all the viewpoints of MLPs.[33] The survey completion rate was low, representing approximately 2% of total ASCLS membership.

Some strengths include that this study provided person-level data from MLPs working in diverse geographical areas in the United States and a variety of clinical laboratory specialties. It also allowed for individual laboratory professionals to express the impact that COVID-19 was having on their work environment in real time, compared with a few months earlier, before the pandemic was declared. More research is needed to measure longitudinal changes during this unprecedented surge in the need for adequately trained laboratorians and sufficiently equipped clinical laboratory services.

Conclusion

Our study identified key changes experienced by medical-laboratory professionals during the initial months of the SARS-CoV-2 outbreak. CDC policies are meant to control spread of COVID-19 in the workplace. However, these policies did not address continued laboratory staffing shortages or reduce demands on existing staff members to incorporate new SARS-CoV-2 detection technologies into the testing menu. Further, public health guidelines cannot fully address concerns about safe social distancing in clinical laboratories, and many MLPs fear they may become ill with COVID-19 through exposure at work. Positive ratings for the response to the pandemic by health care facilities and laboratory leadership influenced MLP job satisfaction, feelings toward their work, and laboratory morale.

Although MLPs receiving in-kind gifts and recognition at their workplace influenced positive feelings toward their work, laboratories reportedly implemented cost-reducing measures and appeared unable to provide monetary compensation to MLPs. This circumstance may be due to lack of relief funds from the government and concerns about reduced revenue associated with a drop in the provision of routine health care services, which are issues that are likely to continue in the next few months, even as the COVID-19 vaccination campaign gets underway.

Finally, policies to limit test utilization for COVID-19 may have helped manage a surge in diagnostic laboratory testing. When a pandemic is ongoing, we need not only trained scientists but also individuals who possess the knowledge and flexibility to perform complex clinical laboratory tests for the purpose of diagnosis and treatment, quickly and on a massive scale. However, test management and an able workforce, in the absence of a federally coordinated distribution of necessary reagents and supplies, cannot fully address the increased demand for COVID-19 tests.

We know that MLPs can readily produce millions of tests very quickly, but the strain placed on laboratory management, including availability of supplies and inability to provide additional pay, can impact MLP job satisfaction, feelings toward their work, and morale at the workplace. Successful management and scaling up of COVID-19 diagnostic testing will remain a challenge for clinical laboratories if shortages of needed resources are not addressed. **LM**

Acknowledgements

We recognize support toward the completion of doctoral work from the KU Endowment and support from the KU Clinical Laboratory Science department. We thank the dedicated laboratory professionals who responded to our survey while working in the front lines during the SARS-CoV-2 pandemic, and to all who have worked through this time to care for others. We are also thankful for the input from the reviewers, whose comments were invaluable in achieving the final version of this manuscript. Finally, we also thank Brad Pfaltzgraff, MLS(ASCP)^{CM}MB^{CM}, for his insightful collaboration.

Personal and Professional Conflicts of Interest:

None reported.

Disclosure: The findings and conclusions are those of the authors and do not necessarily represent the official policies and positions of the University of Kansas Medical Center or The University of Kansas Health System.

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