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Competitive Bidding – the Continuing Saga

PAULA GARROTT

Competitive bidding is an approach that has long been favored by Congress to attempt to control costs in a variety of arenas by allowing the marketplace to competitively determine prices. Some would argue it is, on the surface, the “American Way”. However, each time this approach has been suggested to control the cost of laboratory services the clinical laboratory community has staunchly argued that while competitive bidding for durable goods may be effective, it will not work for services that are provided by highly skilled professionals in diverse settings to even more diverse populations.

For many years a competitive bidding strategy was discussed by Congress, but the laboratory community effectively lobbied against it. However in 2003, in the wake of public pressure over provision of prescription drugs for Medicare recipients, the Medicare Prescription Drug Improvement and Modernization Act (MMA) was passed by Congress and included a mandate for a competitive bidding demonstration project for clinical laboratory services. The proposed purpose of the project was to determine if market driven competitive bidding could be used to decrease costs to the Medicare program to provide quality clinical laboratory services. Although the project was to begin in April 2007, the complexities involved in the development of bidding guidelines have delayed the implementation.

The current mechanism for reimbursing laboratories for services provided to Medicare beneficiaries is a fee schedule that was established in 1984. Although most laboratory stakeholders recognize that the fee schedule is outdated and irrational based on today’s technology, they are united in their belief that competitive bidding will be disastrous for the laboratory community as a whole and, more importantly, for the patients and the healthcare providers they serve. The Spring 2007 Washington Beat column enumerated the reasons the clinical laboratory industry opposes competitive bidding

The Clinical Laboratory Coalition (CLC), a group which represents a large number of laboratory stakeholders including ASCLS, has worked to educate members of Congress regarding the potentially negative impact of competitive bidding and to suggest more viable mechanisms for updating the laboratory fee schedule. In addition, they have attempted to work with the Centers for Medicare and Medicaid Services (CMS) to provide feedback regarding the proposed demonstration project.

In early July, CMS released the Medicare clinical laboratory competitive bidding demonstration project draft bidders’ package (available on the CMS website: www.cms.hhs.gov) which contains specific information regarding who will be required to bid and the bidding guidelines. CMS conducted an open door forum on July 16 in Baltimore, Maryland to discuss the draft bidders’ package. The timing of the open door forum coincided with another CMS meeting on coding and the ASCLS and AACC Annual Meetings and Laboratory Exposition, making participation by many stakeholders difficult. ASCLS Executive Vice President Elissa Passiment and Government Affairs Committee Chair Judy Davis participated from the ASCLS meeting site by phone.

The draft bidders’ package left many questions unanswered. Due to the escalating concerns of the laboratory industry, and particularly by small laboratories, the House of Representatives Committee on Small Business conducted a hearing on July 25, 2007. Committee members heard testimony that resulted in committee chairwoman, Nydia Velazquez of New York, concluding that if the competitive bidding project moves forward as designed it could result in small laboratories being hindered or excluded from providing timely, quality laboratory services. This would have an adverse effect on some of the most vulnerable patients in small communities, in rural settings, and in skilled nursing facilities.

As a result of the hearing on August 4, 2007 Representative Velazquez introduced HR 3453, the Community Clinical Laboratory Fairness in Competition Act of 2007, which

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Washington Beat is intended to provide a timely synopsis of activity in the nation’s capitol of importance to clinical laboratory practitioners. This section is coordinated by Paula Garrott, Co-chair of the ASCLS Government Affairs Committee; and Don Lavanty, ASCLS Legislative Counsel. Direct all inquiries to ASCLS, (301) 657-2768 ext. 3022, (301) 657-2909 (fax); or mail to ASCLS, 6701 Democracy Boulevard, Suite 300, Bethesda MD 20817, attn: Washington Beat.
amends title XVIII of the Social Security Act to repeal the Medicare competitive bidding demonstration project for clinical laboratory services.

At the time of the writing of this article, HR 3453 has been referred to the Committee on Energy and Commerce, and in addition to the Committees on Ways and Means, the Judiciary, and Small Business. There is currently no companion bill in the Senate. ASCLS members have been asked to contact their representatives to urge them to support and sign on to HR 3453.

Meanwhile, CMS is proceeding with plans to implement the clinical laboratory competitive bidding demonstration project by spring of next year. The first demonstration site is to be announced this fall. The final bidders' package is also to be released this fall with bids due presumably before the end of the year.
Respect and Responsibility...  
with Apologies to Jane Austen  

SUSAN J LECLAIR

This past May, as our department held its annual pre-commencement robing ceremony, I was struck not for the first time with the importance of the ASCLS Code of Ethics. While we use a shortened version for the ceremony, the clarity of a professional's duty to the patient, to the profession, and to society struck me as more than just words. Since this is my last editorial, I ask your indulgence for some philosophical musing.

Our first duty is to the patient, not to the hospital or the physicians or the insurance companies and I believe that, as individuals, we truly understand this. But how often does this primacy become lost in the paperwork and the machinations of the healthcare system? How often do we begin to think of the 100th CBC or the next urine culture instead of the person from whom these specimens were taken? Should we be so content to increase our workload without assessing how quantity can impact quality? Have we taken an active role in assessing and maintaining quality in ourselves and in our own facilities? How often do we instruct on-the-job trainees just so results can get out without thinking about the quality of those results? Do physicians train people who didn’t go to medical school?

Over the years, one of the most common complaints I have heard from laboratory practitioners is a variation of Rodney Dangerfield’s “I don’t get no respect”. Do we respect other laboratory practitioners, especially those on other shifts? Do we welcome new employees and make them feel comfortable? Do we dress and act like the other professionals we acknowledge every day? Do the folks who dress in scrubs in order to not soil their own clothes really mean that they do not trust their own technique? Do faculty at programs in clinical laboratory science engender activism in this profession? Do they teach and model professional behavior as a way of life?

Many find more problematic than the first two components of the Code of Ethics the third section that speaks to a duty to community. For too long laboratory practitioners have been content to reside behind the conclusions of a single small study that suggested we were introverted and unwilling to deal with confrontation. For too many reasons, our society is in trouble. At present it is both anti-intellectual and anti-science. If we are to be believed in our assertion that we are scientists and not just recipe-followers, then we need to reach out to our communities to explain and defend science. Do we present topics of interest to the local cancer society or speak at town meetings on the benefits of recycling? Do we explain the difference between a matter of personal opinion and scientific theory to local students? Do we judge science fairs? Do we participate in issue-based or political campaigns? Do we vote? Current polls suggest that we, along with the rest of Americans, do not. Yet we bear a higher responsibility than some others in this scientifically and technologically-based society. We are scientists. We know better than to be silent.

Thank you for reading and thank you for the honor of being editor-in-chief.

Susan J Leclair PhD has served as editor-in-chief of Clinical Laboratory Science since 2000.

Clin Lab Sci encourages readers to respond with thoughts, questions, or comments regarding this article. Email responses to ic.ink@mchsi.com. In the subject line, please type “CLIN LAB SCI 20(4) DD LECLAIR”. Selected responses will appear in the Dialogue and Discussion section in a future issue. Responses may be edited for length and clarity. We look forward to hearing from you.
ASCLS Code of Ethics Preamble The Code of Ethics of the American Society for Clinical Laboratory Science (ASCLS) sets forth the principles and standards by which clinical laboratory professionals practice their profession.

I. Duty to the Patient Clinical laboratory professionals are accountable for the quality and integrity of the laboratory services they provide. This obligation includes maintaining individual competence in judgement and performance and striving to safeguard the patient from incompetent or illegal practice by others.

Clinical laboratory professionals maintain high standards of practice. They exercise sound judgment in establishing, performing and evaluating laboratory testing.

Clinical laboratory professionals maintain strict confidentiality of patient information and test results. They safeguard the dignity and privacy of patients and provide accurate information to other healthcare professionals about the services they provide.

II. Duty to Colleagues and the Profession Clinical laboratory professionals uphold and maintain the dignity and respect of our profession and strive to maintain a reputation of honesty, integrity and reliability. They contribute to the advancement of the profession by improving the body of knowledge, adopting scientific advances that benefit the patient, maintaining high standards of practice and education, and seeking fair socioeconomic working conditions for members of the profession.

Clinical laboratory professionals actively strive to establish cooperative and respectful working relationships with other healthcare professionals with the primary objective of ensuring a high standard of care for the patients they serve.

III. Duty to Society As practitioners of an autonomous profession, clinical laboratory professionals have the responsibility to contribute from their sphere of professional competence to the general well being of the community.

Clinical laboratory professionals comply with relevant laws and regulations pertaining to the practice of clinical laboratory science and actively seek, within the dictates of their consciences, to change those which do not meet the high standards of care and practice to which the profession is committed.
The Doctorate in Clinical Laboratory Science: A Projection of Professional Outcomes

ELIZABETH KENIMER LEIBACH

ABBREVIATIONS: ASCLS = American Society for Clinical Laboratory Science; BOD = Board of Directors; CLS = clinical laboratory science; DRC = doctorate review committee; DCLS = doctorate in clinical laboratory science; GTF = graduate task force; PD = professional doctorate; PDTF = Professional Doctorate Task Force

INDEX TERMS: clinical doctorate; clinical laboratory science; evidence-based practice; professional doctorate.


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At the meeting in conjunction with the 2007 ASCLS in San Diego, California, the Board of Directors (BOD) approved a recommendation from the Professional Doctorate Task Force (PDTF) as follows:

“That the ASCLS Board of Directors establish a transition committee during 2007-2008 to include members from the Professional Doctorate Task Force to oversee the continuing development of DCLS programs and practice, as well as the creation of guidelines defining a formal, statutory body to assume responsibility for the permanent oversight of advanced laboratory practice, certification, and recognition.”

This single step by the governing body of ASCLS has firmly set the direction of clinical laboratory science for the foreseeable future. This direction has placed the responsibility of leadership of the profession with the doctorate, the keystone clinical laboratory science (CLS) practitioner.1 The purpose of this article is to discuss the projected professional ramifications of this direction. The professional doctorate cycle below serves as a useful organizing construct for this discussion.

As defined elsewhere, progress in the development of the steps in this professional doctorate (PD) cycle process, though displayed as linear, will occur concurrently, at different rates of development, and with a changing focus depending on (sometimes unforeseen) environmental influences, e.g., CMS regulations interpretations, competitive bidding development, changes in the scopes of practice of other healthcare professions.2

The ASCLS PDTF and the NAACLS Graduate Task Force (GTF) began the PD cycle developmental process during the years 2005-2007. A national debate was entertained that resulted in the conceptualization of the professional doctorate, the doctorate in clinical laboratory science (DCLS), as the keystone practitioner of the profession and, as such, responsible for leading in professional issues strategy development related to research, education, and practice. Collaboratively, the two task forces addressed different aspects of PD educational program development. The PDTF developed competencies and curriculum while the GTF developed educational program standards. Educational program standards for the DCLS are available from NAACLS. In addition, a NAACLS Doctorate Review Committee (DRC) has been established to review accreditation applications. The curriculum materials have been distributed for external (non-CLS review) and feedback has been received from a majority of reviewers. Internal reviewers (representatives from each ASCLS scientific assembly section) have been identified, but material distribution to these individuals, as well as consideration of revisions suggested by the external reviewers, await appointment of the professional doctorate transition committee as approved by the ASCLS BOD in July 2007. Included for your preview, however, is Table 1 which contains the curriculum content areas for which course descriptions and instructional/learning
objectives have been developed. Crowning the collaboration of the profession and the accrediting body is the development and public distribution of the Educational Statement Regarding Doctoral Level Clinical Laboratory Science Professionals. This concise statement summarizes the vision for the DCLS and will be circulated within clinical laboratory professional organizations and to the public at large. These advances represent the initiation of Steps 1 and 2 in the PD cycle.

Activities supporting Step 3 began with construction of the DCLS program implementation resources questionnaire, a comprehensive national survey for identification of resources available for DCLS program implementation. The DCLS program resources questionnaire may be completed and submitted online. The link to the online survey was distributed through the CLS educators' listserv, and to institutional representatives attending the First and/or Second Professional Doctorate Implementation Forum(s) August 26, 2007 with a response requested by September 30, 2007. If you would like to receive the link and complete the survey for your institution (one response per institution), contact Dr. Elizabeth Kenimer Leibach at ekenimer@mcg.edu. The hope is that through analysis of survey responses, resources can be located nationally to provide a pool for collaborative DCLS program delivery.

During the Second Professional Doctorate Implementation Forum held in conjunction with the 2007 ASCLS in San Diego, four possible models for national collaboration were discussed and are summarized in Table 2. These program delivery options represent a continuum from total institutional independence in delivery and degree-granting (first option) to complete sharing of program resources and degree-granting (last option). Considerations as to which model best fits a particular institution interested in program implementation should include not only complexities related to program proposal and implementation in that institution, but also politics and cost assessments involved in partnering with separately governed and accredited institutions. For example, Table 3 is a matrix of relative costs associated with each collaborative model, considering proposal development (to include external accreditation issues), institution and system review, available academic resources, student support, and instructional delivery strategies. This matrix suggests (but individual assessments can differ) that the most cost-effective way to deliver collaborative DCLS instruction among different, partnering institutions may be through shared course content development with independent institutional delivery (the lowest relative costs per institution).

Steps 4 and 5 of the PD cycle have not formally begun and their timely commencements are crucial to the establishment, validation, and acceptance of the DCLS. “Practitioner Recognition” includes not only the graduation of DCLS practitioners but also data collection to support evaluation of DCLS contributions to and impact on healthcare delivery and to support job analyses for credentialing. With “Practitioner Integration” will come generation of evidence to be utilized in practice, analyzed to improve educational programs, and communicated throughout the profession and the entire healthcare delivery system. Integration of the DCLS in healthcare delivery will potentially coalesce disparate aspects of the profession (e.g., education, research, practice, administration, manufacturing, regulatory) into one voice for quality for the profession based on evidence of increased positive patient outcomes, cost-effectiveness, and safety.

However, there is still concern among some specialties within the profession that the DCLS is not a professional “solution”

### Table 1. DCLS curriculum content areas

<table>
<thead>
<tr>
<th>Group</th>
<th>Content Areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Advanced basic sciences</td>
</tr>
<tr>
<td>Group II</td>
<td>Patient interactions</td>
</tr>
<tr>
<td>Group III</td>
<td>Clinical laboratory diagnosis and therapies</td>
</tr>
<tr>
<td>Group IV</td>
<td>Applied statistics, research methods, evidence-based practice</td>
</tr>
<tr>
<td>Group V</td>
<td>Education, ethics, policy, and clinical services delivery</td>
</tr>
</tbody>
</table>

### Figure 1. The professional doctorate (PD) cycle
but in fact a potential professional “problem” even though the gap in healthcare delivery that the DCLS will address has been defined,4 interviews with CLS advanced practitioners to collect data describing functions of the DCLS are underway, and many potential DCLS program applicants have self-identified. Among CLS clinical administrators particularly, there is fear that introduction of the DCLS will negatively impact an already dwindling baccalaureate-level workforce. Responses range from actively opposing existing and proposed state licensing and certification requirements to beginning hospital-based certificate programs in competition with established degree-based programs. The counterpoint to those responses is to begin Steps 4 and 5 by forging partnerships among educational institutions, professional organizations, clinical providers, and industry stakeholders for the purpose of solidifying support for the DCLS. Supporting the DCLS industry-wide will signal to all of healthcare that the CLS profession is unified in the intention of managing quality of laboratory information for the benefit of the public good, and as a profession, is willing to define education, research, and practice (at all career levels) to support that intention. Together representatives from all quarters of the CLS industry can begin crafting strategies for the collection, evaluation, and publication of evidence of the impact of clinical laboratory information and begin designing processes to document the outcomes of this information that the DCLS will, in professional practice, continue to develop, refine, and report.

Considering the venue in which representative professional stakeholders will discuss practitioner recognition and integration will complete discussion of the purpose of this article:

…but to consider the professional ramifications of ASCLS BOD approval of a ‘transition committee to oversee the continuing development of DCLS

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**Table 2. DCLS program delivery models**

1. Independent institutional development and delivery
2. Shared course content development with independent delivery
3. Shared course development and delivery with independent degree granted
4. Shared curriculum and consortium degree granted

**Table 3. Cost considerations of DCLS program delivery models**

<table>
<thead>
<tr>
<th>RELATIVE COSTS PER INSTITUTION ASSOCIATED WITH:</th>
<th>Individual delivery¹</th>
<th>Shared courses²</th>
<th>Common delivery³</th>
<th>Consortium degree⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROGRAM PROPOSAL Prescribed institutional format</td>
<td>$$$</td>
<td>$</td>
<td>$</td>
<td>$$$</td>
</tr>
<tr>
<td>Institutional review</td>
<td>$</td>
<td>$</td>
<td>$$$</td>
<td>$$$</td>
</tr>
<tr>
<td>System review</td>
<td>$</td>
<td>$</td>
<td>$$$</td>
<td>$$$</td>
</tr>
<tr>
<td>PROGRAM IMPLEMENTATION Academic resources</td>
<td>$$$</td>
<td>$$$</td>
<td>$</td>
<td>$$$</td>
</tr>
<tr>
<td>Student support</td>
<td>$$$</td>
<td>$$$</td>
<td>$$$</td>
<td>$$$</td>
</tr>
<tr>
<td>Instructional delivery</td>
<td>$$$</td>
<td>$$$</td>
<td>$$$</td>
<td>$$$</td>
</tr>
<tr>
<td>TOTAL $</td>
<td>19</td>
<td>17</td>
<td>18</td>
<td>24</td>
</tr>
</tbody>
</table>

1. Independent institutional development and delivery; 2. Shared course content development with independent delivery; 3. Shared course development and delivery with independent degree granted; 4. Shared curriculum and consortium degree granted
programs and practice, as well as the creation of guidelines defining a formal, statutory body to assume responsibility for the permanent oversight of advanced laboratory practice, certification, and recognition.”

PDTF and GTF prescience in planning foresaw the need for permanent oversight of all doctoral-level activities, i.e., leadership of the profession through direction in education, research, and practice.2 The ASCLS transition committee is responsible for developing guidelines to establish the formal statutory body that will oversee these aspects of our advancing practice. The clinical laboratory, by every cost, revenue, and quality measure, is a huge force in healthcare and, ergo, the divisive elements struggling for control of that force are formidable. The mission before the CLS profession is to unify the direction of the industry by focusing on quality in the generation and utilization of laboratory information – and evaluating, documenting, and communicating the impact of education, licensure and certification, workforce, technology, and reimbursement in facilitating the attainment of these goals. With the understanding that DCLS development and practice will define the career ladder and practice for the entire profession, the academy will serve also as the venue for professional dialogue among industry partners regarding coalescing of support and a unity that will move the profession forward.

Continue to monitor our professional literature and the ASCLS website (www.ascls.org) for progress updates on the structure, function, and membership of the academy and for the latest developments in implementation of the professional doctorate. Please post general comments to the ASCLS forums. (You can find the forums from the “About” link on the title bar of the ASCLS homepage). Your opinions, interest, and support are vital!

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LETTER FROM THE EDITOR

Transitions

SUSAN J LECLAIR

It is with great pleasure that I introduce the incoming editor-in-chief of Clinical Laboratory Science, David Fowler PhD CLS(NCA). Most of you already know David from his many and varied activities with ASCLS. A past president of ASCLS, David provided the creative thrust behind the doctorate in clinical laboratory science (DCLS) discussions and planning. Most recently he has served as editor for Research and Reports section. His term will begin with the January 2008 issue.

On a personal note, I wish to thank everyone with whom I worked for the past two terms as editor-in-chief. To work for an award-winning journal is an honored activity; to work with individuals who live the ideal of professionalism is a joy. Thank you all.

Susan J Leclair PhD has served as editor-in-chief of Clinical Laboratory Science since 2000.
The Rural Rotation in a Medical Technology Program:
A Ten-year Retrospective Study

BEVERLY A KIRBY

OBJECTIVE: This study evaluated the effectiveness of a rural rotation as a tool to recruit medical technology program graduates to medically underserved areas.

DESIGN: A paper survey was distributed to all 1994 – 2003 graduates of the West Virginia University Medical Technology Program.

SETTING: The survey was mailed to the graduates’ homes.

PATIENTS OR OTHER PARTICIPANTS: Ninety-four of the two hundred six surveys were returned for a response rate of 45.6%.

INTERVENTIONS: Surveys were mailed in January 2004.

MAIN OUTCOME MEASURES: Responses to questions regarding choice of site for rural rotation, whether or not a job was offered at the rural site, and whether the graduate subsequently worked at a rural site were tabulated. Responses to questions concerning whether the rotation helped the respondent to appreciate the needs of rural health facilities and whether the rotation resulted in a greater interest in working in a rural area were tabulated. Responses were also tabulated for questions about the value of the rural rotation to the respondent’s education and whether the rural rotation was recommended for future students.

RESULTS: Of respondents, 70.2% chose their rural rotation sites due to proximity to their homes and 38.3% were offered jobs at their rural rotation sites. 50% of all respondents subsequently worked at a rural site. Of respondents, 73.4% indicated strong agreement that the rotation helped them appreciate the needs of the rural facility, and 37.2% indicated agreement with becoming more interested in working at a rural site. Of respondents, 65% indicated that the rural rotation was beneficial and that they would recommend it to future students.

CONCLUSION: Results of the survey suggest that a prior rural affinity is a factor associated with selection of rural sites for medical technology program graduates. The survey results also suggest that a rural rotation during medical technology education is beneficial to individuals, including those who elect not to go to rural sites after graduation.

ABBREVIATIONS: Health professions shortage areas (HP-SAs) nurse practitioners (NP) medically underserved areas (MUAs) West Virginia Rural Health Education Partnerships (WVRHEP).

INDEX TERMS: education; medically underserved; recruitment; rural rotation.

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West Virginia is a predominantly rural state with 64% of its population living in communities of less than 2500 individuals. The only two cities with populations over 50,000 are Charleston and Huntington. Of the state’s 1,808,344 people, 15.3% are over 65 years old compared to the national average of 12.4%. In 1999, 17.9% of the state’s population lived below the poverty level compared to the national average of 12.4%. All but eight of West Virginia’s fifty-five counties are designated as medically underserved areas (MUAs) and forty-four of these counties are federally designated as health
professions shortage areas (HPSAs). West Virginia’s aging population, poverty, rough terrain, and lack of available medical care result in West Virginians having more health problems than people of other states. The following leading causes of death have age-adjusted rates that are consistently higher than the national rates: heart disease, malignant neoplasms, cerebrovascular disease, chronic obstructive pulmonary disease, accidents, pneumonia and influenza, diabetes mellitus, and suicide. These statistics are all according to the annual report of the West Virginia Department of Health and Human Resources.  

In 1991 the West Virginia University system received a four year Kellogg Community Partnership grant to develop a community-based academic program to address the issues of recruiting, preparing, and retaining health professionals for the rural areas of the state. During the same time, the governor called a special legislative session which resulted in the passage of H.B. 213, the Rural Health Initiative Act. The partnership of these two initiatives resulted in a tremendous infrastructure for educating health professionals with the vision of alleviating the shortage of medical care for the citizens of West Virginia.

Legislation in 1995 integrated the Rural Health Initiative and the Kellogg Community Partnerships programs into the West Virginia Rural Health Education Partnerships (WVRHEP). The program has grown into a network of 13 consortia covering 47 counties with 255 training sites and 493 field faculty. The health profession programs’ curricula were amended to include required rotations in rural communities comprised of discipline-specific clinical training, interdisciplinary case management, and community service and/or community-based research.

To establish an educational pipeline, the W.K. Kellogg Foundation developed and funded the Health Sciences and Technology Academy to increase the number of economically disadvantaged and African American high school students who pursue post-secondary education in the health sciences. Additionally, the West Virginia Recruitment and Retention Project was funded by the Claude Worthington Benedum Foundation to provide financial incentives and placement activities.

Continued legislative funding reflects the state commitment to the WVRHEP vision: “In West Virginia, we envision a time when all residents of our most underserved, rural communities have local access to high-quality primary healthcare provided by well-trained, high quality healthcare professionals.”

The West Virginia University Medical Technology Program officially became involved in the state rural health initiative with a federal grant for the interdisciplinary training of health professionals. The Rural Interdisciplinary Training Grant provided for a collaborative training program of medical technology, physical therapy, and social work students. The grant funded one-month rural rotations for students for three years beginning in 1993. Participating students received stipends as well as free housing. The resultant curricular changes remained in effect beyond the funding to allow a mandatory one-month rural rotation for every medical technology student. Students no longer receive stipends but are eligible for free housing at approved RHEP sites.

The current nationwide shortage of medical technologists is especially critical in the rural areas. Small hospital laboratory managers are increasingly frustrated by the inability to attract and retain laboratory professionals. The medical technology program at West Virginia University envisions the required rural rotation as a recruitment and retention tool that makes rural practices a more attractive choice for healthcare practitioners.

Previous studies of health professionals have demonstrated factors that appear to have a positive effect on the choice of medically underserved areas for practice sites. The most frequently studied question has been whether exposure to medically underserved areas during training significantly influences the eventual choice of practice location. Tavernier, Conner, Gates, and Wan surveyed 775 physicians within three to six months of completing a family medicine residency in an attempt to evaluate the effects of exposure to underserved areas during training. They defined MUAs as rural communities with population of 20,000 or less at least 60 miles from any metropolitan area with a population of at least 100,000; inner city areas with a majority of population being of low socio-economic status; or international third world countries. 3 As expected, those participants who had exposure to rural communities prior to their medical training demonstrated an increased desire to locate to an MUA. The findings, however, also demonstrated the positive effects of early exposure during training on later practice site choice. The association of early service experience participation was very similar to being born and/or raised in an MUA. 3

Recognizing the importance of prior exposure to rural areas, several medical education programs have made an attempt to recruit students into their programs based on their rural
background. The University of Louisville reported a successful project that identified students from rural areas who were interested in health professions and involved the students in labor-intensive programs to increase their ACT scores. This resulted in an increased pool of applicants to medical school with affinity for rural practice. The University of Nebraska Medical Center has also recognized the importance of recruiting students who have a previous affinity for rural practice. They are making a concerted effort to identify and accept students who have a rural orientation.

The University of Adelaide, South Australia reviewed twelve studies and concluded that "there is consistent evidence that the likelihood of working in rural practice is approximately twice greater among doctors with a rural background". They further concluded that "there is a smaller body of evidence in support of the other rural factors studied, and the strength of association is similar to that for rural background". The other rural factors included rural schooling, having a rural partner, rural undergraduate training, and rural postgraduate training.

The University of Arkansas examined factors and incentives promoting long-term employment of nurse practitioners (NP) in rural Arkansas. Their evidence indicated that NP students who participated in clinical practicums were more likely to practice in rural areas.

The Te Waipounami Rural Health Unit of the University of Dunedin, New Zealand evaluated the effect of a fifth-year rural health curriculum on the attitudes of students toward a career in rural general practice. The investigators found that students who came from rural backgrounds are more likely to have a positive attitude toward rural general practice. They found, however, that a rural curriculum can produce attitude changes in other students. They concluded, therefore, that medical schools should address the needs of rural communities by selecting students of rural origin and also ensuring a significant rural health component of the curriculum.

A ten-year retrospective study of the rural experience of students in the Medical Technology Program at West Virginia University is reported here. Because the primary goal of the required rural rotation is recruitment and retention of laboratory professionals in the MUAs of West Virginia, it is important to determine whether the rural rotation is having a positive effect on the likelihood of participants' working in rural areas upon graduation.

METHOD
Participants
All 206 graduates of the West Virginia University Medical Technology Program from 1994 to 2003 were invited to participate. All program graduates during that time had been required to complete an enrichment rotation. The majority of the enrichment rotations had been completed at rural sites, although some students had been permitted to complete their rotations at small community hospitals in non-rural areas.

Materials
A cover letter invited participation and assured the participants of their anonymity (Appendix A). A self-addressed postage-paid envelope was provided.

The medical technology program rural health education coordinator designed a questionnaire to obtain information regarding where the rural rotation was completed, why the site was chosen, and whether or not the respondent subsequently worked at that site or any other medically underserved site. Four questions regarding the respondent’s opinion of the rural rotation were rated by a Likert scale and the respondent was also invited to provide comments (Appendix B).

RESULTS
Of 206 questionnaires sent out, 94 were completed and returned for a 45.6% response rate. Sixty-six (70.2%) of respondents gave nearness to home as their reason for choice of rotation site. Fifteen (16.0%) stated that they chose the site because it was near friends or family members. Two (2.1%) stated that the site was in an area of interest. Three (3.2%) stated that they were placed at the site by the program director. Two (2.1%) chose the site because of housing availability. The remaining respondents did not give a specific reason for their choice (Figure 1).

Of the 66 participants who indicated that their rural rotation sites were chosen because they were close to home, only 28 (42.4%) indicated that they worked at a rural site. Thirty-eight (57.6%) indicated that they had not worked at a rural site after graduation, but five of these indicated that they were currently continuing their education in medical school, dental school, physicians’ assistant programs, or other forms of graduate education.

Of the 66 participants who indicated that they chose their rural rotation sites because they were close to their homes, 51 (77.3%) indicated through a positive response (4 or 5)
that they appreciate the needs of rural hospitals or clinics. Only 15 respondents of this group responded with a neutral or negative rating (Figure 2).

Twenty-five of the 66 participants (37.9%) who indicated that they chose rural rotation sites because they were close to home indicated that the rural rotation made them more interested in working at a rural site but 41 of them (62.1%) gave neutral or negative responses (Figure 3).

Of those 69 participants who rated their appreciation of the needs of rural hospitals as high, 51 (73.9%) had also indicated that they chose their rural rotation sites because they were close to home (Figure 4).

Of those 52 participants who rated their level of interest in working in a rural area as high, 45 (87%) had also indicated that they chose their rural rotation sites because they were close to home (Figure 5).

Of those 47 participants who have worked in rural areas since graduation, 31 (66%) are those who also chose their rural rotations because they were close to home.

Thirty-six of the 94 respondents (38.3%) indicated that they were offered jobs at their rotation sites. The other 58 (61.7%) indicated that they were not offered jobs.

Forty-seven of the 94 respondents (50%) stated that they had worked at that site or at another medically underserved site after graduation. The other 47 (50%) indicated that they had not worked at an underserved site.

Figure 6 represents all responses regarding whether the rotation helped the respondent appreciate the needs of rural hospitals or clinics. One respondent wrote “NA”.

Figure 7 represents all responses regarding whether the rural rotation made the respondent more interested in working at a rural site after graduation. One respondent wrote “NA” and one did not answer.

The 94 respondents also replied to the question of whether the rural rotation was beneficial to the education at West Virginia University with the following results (Figure 8).

The 94 respondents were also asked to rate whether they recommended the rural rotation for future students (Figure 9).
DISCUSSION

The results of the questionnaire appear to support the value of the rural rotation to the goal of recruiting and retaining laboratory professionals to the rural areas of West Virginia. Approximately one-third of the graduates were offered positions at the site where they did their rotation and half of the respondents ended up working in medically underserved areas. Sixty-five percent of students indicated that they recommended rural rotations for future students and also indicated that they felt that the rotation had been beneficial to their education.

The qualitative data that resulted from the open-ended questions added a richness to the evaluation of the rural rotation program. The graduates offered some valuable insights into the benefits of the experience. Even the students who have chosen not to work in rural areas suggested that they were enriched by the experience, as can be seen in the following representative quotes:

“The rural rotation was an eye opener. We were told about smaller institutions and the technology that was there or not there, but to see it was completely different. The rural rotation is a great experience for students getting ready to enter the job force.”

“Rural rotation was valuable for me as a student. I feel students need to be exposed to as many environments as possible. I chose to work in an urban area after graduation, but that doesn’t mean the rural rotation wasn’t valuable to me. I also feel that the rotation would be beneficial to future students at WVU but should be a mainstay in programs across the country.”

“Rural hospitals are such a different experience! Never thought I would work in a rural area, but I have X3!”

“I loved the rural rotation. It made me realize that there are still hospitals that are not computerized. Though I work at a large facility now, I feel like I would like someday to go to a smaller setting and I feel that the rural rotation gave me a little insight into that.”

“I feel the rural rotation provides beneficial, hands-on experience for students. I, however, prefer a more fast-paced work environment. Thus, a rural hospital is not the place for me.”

“Medical technologists are desperately needed in rural areas of the state. Some facilities only hire BS med techs and
they are very hard to find. I would highly recommend students trying rural rotations in the southern part of the state. Maybe it would spark a job interest and help with our med tech shortage.”

“My rural rotation was a truly wonderful experience. It helped me to really put my skills to work in a guided situation other than in school.”

Students who chose their rural rotation sites because they were close to home offer some insight supporting the value of early exposure to rural environment in recruiting to rural areas. Those students who were from rural areas appear to be more likely to return to rural areas to work and to appreciate the unique needs of rural hospitals. This supports the ideas of recruiting and preparing students from rural areas to enter the healthcare professional programs.3,6 Those who already have rural affinity are more likely to furnish the medical manpower to the state’s medically underserved areas.

The results of this survey support continuing to require rural rotations of all medical technology students at West Virginia University. The rotation appears to be associated with some degree of success in recruitment and retention of medical technologists in underserved areas. It also appears to be beneficial to individuals who do not choose to work in rural areas. The survey supported the decision to require more actual rural exposure as part of the rotation. As a result, all current and future students are encouraged to participate fully in the community and interdisciplinary opportunities at the rural sites. Students who are not close to home are also being encouraged to select sites that provide housing so that they can become more involved in the communities. It is expected that this will enhance the enrichment experience for participants.

CONCLUSION
The required rural rotation at West Virginia University is a valuable enrichment experience for medical technology students. It serves as a site for applying their clinical skills in a unique way to prepare them for the workforce. It also serves as a recruitment tool for the medically underserved areas of the state.

Exposure to the rural environment serves to enhance an understanding of the needs of a medically underserved population. The study results support the notion that providing that exposure as part of the training of healthcare professionals as well as recruiting and retaining those individuals who already have rural affinity may help alleviate medical personnel shortages.
Clin Lab Sci encourages readers to respond with thoughts, questions, or comments regarding this article. Email responses to ic.ink@mchsi.com. In the subject line, please type “CLIN LAB SCI 20(4) CP KIRBY”. Selected responses will appear in the Dialogue and Discussion section in a future issue. Responses may be edited for length and clarity. We look forward to hearing from you.

REFERENCES


Appendix A. Solicitation letter

January 5, 2004

Dear Graduate of the Medical Technology Program,

As the rural education coordinator for the Medical Technology Program, I am doing a ten-year retrospective study of the benefit of the rural/enrichment rotation to our students. I would really appreciate your input. Participation in this survey is entirely voluntary.

Attached is a brief questionnaire. Please respond to the questions candidly. You may omit any question that you prefer not to answer. Tabulated results may be used to demonstrate the value of the rural rotation to the state health care system and to students. Data from this survey may be published, but individual and site identifiers will be removed to completely protect anonymity. Please do not put your name on the survey.

Please return your response in the enclosed self-addressed postage-paid envelope.

Thank you very much for your anticipated participation in this valuable study.

Yours truly,

Beverly A Kirby, MA MT(ASCP) CLS(NCA)
Assistant Professor
Appendix B. Student questionnaire

West Virginia University Medical Technology Program
Ten-year Retrospective Study of the Rural Rotation

1. Year of graduation from the Medical Technology Program __________________________

2. Site where rural/enrichment rotation was completed ____________________________

3. Reason for your choice of site __________________________________________________

4. Were you subsequently offered a job at the site? _________________________________

5. Did you subsequently work at that site or any rural site? Keep in mind that all of West Virginia is considered rural or medically underserved except for Morgantown, Charleston and Huntington. ____________________________

6. List places you have worked since graduation. ____________________________________

7. Rate each of the following by circling a number on a scale of 1 – 5 with 1 representing strong disagreement with the statement and 5 representing strong agreement with the statement.

The rural rotation helped me appreciate the needs of rural hospitals and clinics.

1 2 3 4 5

The rural rotation made me more interested in working at a rural site after graduation.

1 2 3 4 5

The rural rotation was beneficial to my education at West Virginia University.

1 2 3 4 5

I would recommend the rural rotation for future students.

1 2 3 4 5

Please use the back of this page for any additional comments concerning the impact or benefit provided by your rural rotation.
Three Alternative Structural Configurations for Phlebotomy: A Comparison of Effectiveness

HEIDI MANNION, TERESA NADDER

OBJECTIVE: This study was designed to compare the effectiveness of three alternative structural configurations for inpatient phlebotomy. It was hypothesized that decentralized was less effective when compared to centralized inpatient phlebotomy.

DESIGN: A non-experimental prospective survey design was conducted at the institution level. Laboratory managers completed an organizational survey and collected data on inpatient blood specimens during a 30-day data collection period.

SETTING/PARTICIPANTS: A random sample (n=31) of hospitals with onsite laboratories in the United States was selected from a database purchased from the Joint Commission on Accreditations of Healthcare Organizations (JCAHO).

MAIN OUTCOME MEASURE: Effectiveness of the blood collection process was measured by the percentage of specimens rejected during the data collection period.

RESULTS: Analysis of variance showed a statistically significant difference in the percentage of specimens rejected for centralized, hybrid, and decentralized phlebotomy configurations \[F(2, 28) = 4.27, \ p = .02\] with an effect size of .23. Post-hoc comparison using Tukey’s HSD indicated that mean percentage of specimens rejected for centralized phlebotomy (M = .045, SD = 0.36) was significantly different from the decentralized configuration (M = 1.42, SD = 0.92, p = .03).

CONCLUSION: Phlebotomy configuration has a significant effect on the percentage of specimens rejected. Based on this outcome, the centralized phlebotomy configuration was found to be more effective when compared to the decentralized configuration.

ABBREVIATIONS: ANOVA = analysis of variance; JCAHO = Joint Commission on Accreditation of Healthcare Organizations; Tukey’s HSD = Tukey’s honestly significant different; LSD = least significant difference; M = mean; SD = standard deviation

INDEX TERMS: decentralized; effectiveness; patient-focused; phlebotomy.


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This study was funded by a Professional Development Grant from the University of Alaska Anchorage and the Allegiance Healthcare Graduate Research Award from the American Society of Clinical Laboratory Science. Results from this study were included in a dissertation submitted by Heidi Mannion in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Health Related Sciences at Virginia Commonwealth University.

In recent years hospitals have utilized many methods to improve the efficiency and quality of their services. In an effort to improve patient satisfaction by reducing delays and the number of health professionals encountered during a hospital stay, some hospitals have implemented a patient-focused care model which includes decentralizing inpatient
phlebotomy services. With this model, nurses and nurse extenders such as patient care technicians and patient care assistants collect the inpatient blood specimens for laboratory testing. Prior to the introduction of patient-focused care models, hospitals utilized a centralized approach, in which the laboratory controlled the management and delivery of phlebotomy services.

Advantages and disadvantages are associated with each type of system for phlebotomy services. Centralized phlebotomy affords reductions in cost from eliminating duplication of effort and resources. A comparison of the systems may indicate that a centralized system carrying out all specimen collection requests for the hospital may be slower than a decentralized arrangement where each individual nursing unit is responsible for its own specimen collections. Decentralized phlebotomy locates the blood collector near the patient allowing flexibility in the blood collection schedule, which may reduce delays in the blood collection process. At the same time, the blood collector’s skill level is difficult to maintain when phlebotomy is performed on an irregular basis and the logistics of phlebotomy training and competency testing becomes more complex with the increased number of personnel performing phlebotomy procedures.1-2

The purpose of this study was to compare alternative structural configurations of inpatient blood collection, centralized, hybrid and decentralized, to determine if equally effective outcomes result from all three systems. In this study, the percentage of specimens deemed unacceptable for analysis by the laboratory (rejected) was used to compare the effectiveness of the three structural configurations. Based on previous studies2-4 it was hypothesized that decentralized inpatient phlebotomy would have a significantly higher percentage of blood specimens rejected than centralized inpatient phlebotomy and be, therefore, less effective.

MATERIALS AND METHODS
This study was conducted at the institution level using a non-experimental prospective survey design. A random sample of 750 hospitals with onsite laboratories in the United States was selected from a database (N = 3454) purchased from the Joint Commission on Accreditation of Healthcare Organizations (JCAHO). Laboratory managers from these institutions were solicited to participate in the study. Facilities that chose to participate signed a consent form, completed an organizational survey, and collected data on inpatient blood specimens that were rejected over a 30-day period. The study was approved by the Institution Review Boards at both Virginia Commonwealth University and the University of Alaska Anchorage.

Due to the low response rate from the first mailing, a second random sample was selected using sampling with replacement. A total of 1,387 letters were sent to solicit participation in the study; 268 (19.3%) facilities responded to the invitation. Prior to selecting a third random sample, the sample size was calculated using the data submitted from three centralized and three decentralized facilities from the first random sample. Minitab software5 was used to perform a power calculation based on an unequal variance t-test. Using a power of .90 with an alpha level of .05, the unequal variance t-test gave a required sample size of six institutions per group. Although 63 facilities agreed to participate, only 31 submitted acceptable data before the close of the study.

Laboratory managers from the random sample that agreed to participate in the study were asked to provide the following organizational information on their facility: 1) average daily census for the hospital during the past six months; 2) average daily census for critical care and pediatric units during the past six months; 3) utilization of students from training programs for inpatient blood collection during the past six months (yes/no); 4) utilization of students from training programs for inpatient phlebotomy service (centralized or decentralized); 5) total cost of phlebotomy consumables for the last fiscal year; 6) total billable inpatient and outpatient blood collections for the last fiscal year; 7) total cost of phlebotomy consumables for the last fiscal year; 8) information contained on specimen labels printed by the laboratory information system; 9) use of hand-held computer technology at the bedside for patient and specimen identification (yes/no); 10) on the job training for inpatient phlebotomy (yes/no), if yes length of training in weeks; 11) educational background of the laboratory manager; 12) method of billing for inpatient blood collections; and 13) use of standard operating procedures for specimen acceptability (yes/no), identify standards if used. In addition to the organizational information, specimen processors were asked to collect data on inpatient specimens rejections over a 30-day period. Reasons for rejecting specimens included: compromised integrity (e.g., hemolyzed, clotted, or contaminated), improper collection, inadequate identification, lab accident (specimen damaged after being received in the lab), lost/damaged during transit, quantity not sufficient for test ordered, and other. Fewer than seven percent of the specimens rejected were due to lab accidents and lost/damage during transit. Compromised integrity
was the major reason for specimen rejection for all three phlebotomy configurations.

Six percent of the respondents indicated that they were unable to classify their phlebotomy configuration based on the description provided in the organizational survey. They estimated that the responsibility for collecting inpatient blood specimens was equally divided among the laboratory (centralized) and nursing services (decentralized). A third category for phlebotomy configuration (hybrid), therefore, was added prior to analyzing the data. Subsequently, the phlebotomy configurations were recoded based on the percentage of laboratory and non-laboratory personnel collecting the inpatient blood specimens. The percentages were calculated using the data collected from the specimen rejection study. The three configurations were defined as: centralized, >80% collected by laboratory personnel; decentralized, >80% collected by non-laboratory personnel; and hybrid, all other facilities.

RESULTS
The sample size requirement was met with 11 centralized (4,219 specimens), 10 hybrid (25,180 specimens) and 10 decentralized (33,449 specimens) facilities included in the analysis. Though a significant difference (p < .01) was found in the average daily census among the three phlebotomy configurations, a significant correlation was not found between the average daily census during the study and the percentage of specimens rejected (r = .10, n = 31, p = .60); therefore, it was not controlled for in the data analysis.

Effectiveness of the blood collection process was measured by the percentage of specimens rejected during the data collection period. Results from the analysis of variance (ANOVA) showed a statistically significant difference in the percentage of specimens rejected for the three phlebotomy configurations, centralized, hybrid, and decentralized [F (2, 28) = 4.27, p = .02] with an effect size of .23. Post-hoc comparison using the least significant difference (LSD) test indicated that the mean for percentage of specimens rejected for centralized phlebotomy (M = 0.45, SD = 0.36) was significantly different from the hybrid (M = 1.22, SD = 1.0, p = .04) and decentralized (M = 1.42, SD = 0.92, p = .01) phlebotomy configurations. The hybrid and decentralized configurations did not differ significantly (p = .59). Moreover, the results from the Tukey’s HSD, a more conservative method, mirrored those of the LSD. A significant difference (p = .03) for the mean percentage of specimens rejected for centralized phlebotomy (M = 0.45, SD = 0.36) was also found when compared to decentralized phlebotomy (M = 1.42, SD = 0.92). The hybrid versus decentralized (p = .85) and hybrid versus centralized
(p = .09) comparisons were not significant. Nonetheless, the results from the latter comparison approached statistical significance. The means plot in Figure 1 demonstrates the differences in percentage of specimens rejected among the three phlebotomy configurations. The data supports the hypothesis that there is a significant difference in the percentage of rejected inpatient blood specimens between centralized and decentralized inpatient phlebotomy services.

A factorial ANOVA was utilized to explore the interactions and effects of the organizational properties measured in the study (phlebotomy configuration, on the job training, and information on labels). With this model there were no significant interactions, and the only significant main effect (p < .01) observed was with phlebotomy configuration. The variable information on labels was not significant and had the lowest effect size; therefore, it was excluded from the analysis and a two-way between groups ANOVA was run. Subsequent ANOVA analysis produced no significant interaction between on the job training and phlebotomy configuration; however, statistically significant main effects were observed for both phlebotomy configuration \([F (2, 25) = 7.474, p < .01]\) and on the job training \([F (1, 25) = 8.563, < .01]\). Furthermore, a large effect size was observed with both variables (partial eta squared = .37 and .26 respectively). For small samples, SPSS provides an adjusted coefficient of determination \((r^2)\), a more conservative estimate of how much variance in the dependent variable is explained by the model. In this model, the adjusted \(r^2 = .33\); therefore, 33% of the variance in the percentage of specimens rejected can be explained by the model.

As can be seen in Figure 2, facilities that provided on the job training have a lower percentage of inpatient blood specimens rejected than facilities that do not provide on the job training. Therefore, on the job training is also associated with a higher level of effectiveness for inpatient phlebotomy services.

**DISCUSSION**

This study found differences in effectiveness when comparing hospitals that utilized centralized, hybrid, and decentralized systems for inpatient phlebotomy services. Centralized phlebotomy services were more effective at providing quality specimens for the laboratory to analyze as reflected in the lower percentage of specimens rejected. Part of this difference may be attributable to the use of phlebotomists and other laboratory personnel who have received training in specimen collection and who understand the impact of the pre-analytical variables on the quality of the tests results produced by the laboratory. In addition to the training, a dedicated phlebotomy team performs inpatient phlebotomy on a regular basis allowing skill level to be maintained. The importance of training is demonstrated in Figure 2; facilities in which the laboratory provided on the job training for their specimen collectors had a lower percentage of specimens rejected across all three phlebotomy configurations.

In this study laboratory personnel collected 92.8% of the inpatient specimens in centralized facilities and nonlaboratory personnel collected 96.5% of the specimens in decentralized facilities. Centralized facilities had a significantly lower (p = .03) percentage of specimens rejected than decentralized facilities. This study supports the findings of several CAP Q-Probes studies which found that nonlaboratory personnel had a significantly higher percentage of specimens rejected than laboratory personnel when comparing aggregate data.

Figure 3 shows no difference in the percentage of specimens rejected for small (< 100 beds) and medium (100-500 beds) sized facilities when a centralized inpatient phlebotomy configuration is used. This was not case for hospitals that

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**Figure 3.** Comparison of percentage of specimens rejected grouped by configuration and bed size

Note: There were no small hospitals that utilized a decentralized configuration.
used a hybrid configuration; smaller hospitals had a higher percentage of specimens rejected than medium-sized hospitals. This may be due to the difficulty in maintaining the specimen collector’s competency when the number of blood collections requested is small. Further, there is an increased number of personnel performing the procedures due to the laboratory and nursing units sharing the responsibility for blood collection. Future research comparing the effectiveness of the three phlebotomy configurations should include the number of phlebotomies performed each day for all personnel responsible for inpatient blood collection.

Results from the study can be generalized only to a population similar to those who responded to the surveys. None of the small hospitals in the study used a decentralized configuration which may have biased the results if this is not reflective of the target population. JCAHO, the owner of the dataset used in the study, was contacted to determine if information on the phlebotomy configurations for the target population was available. They were unable to determine the phlebotomy configurations used by the facilities in their dataset.

Limitations of this study also included the accuracy of the self-reported information on the organizational survey and the interrater reliability of the specimen processors. In addition, the conclusions were based on the analysis of a single measure of effectiveness, percentage of rejected specimens. Therefore, the inclusion of other measures of effectiveness including patient satisfaction and complications from the blood collection process (bruising, nerve damage) are recommended for further studies.

ACKNOWLEDGEMENTS
This study was funded by a Professional Development Grant from the University of Alaska Anchorage and the Allegiance Healthcare Graduate Research Award from the American Society of Clinical Laboratory Science.

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REFERENCES
FOCUS: NEW DIRECTIONS IN HEMOSTASIS AND COAGULATION

Introduction: New Directions in Hemostasis and Coagulation

J LYNNE WILLIAMS

The coagulation laboratory, like other departments in the clinical laboratory, is undergoing major shifts in both the understanding of the pathologic etiology of various diseases and the diagnostic procedures used in the definitive or differential diagnosis of these diseases. Much of this new information comes from advances in the identification of causative genes for the disorders, as well as from implementation of new diagnostic genetic and molecular procedures.

The term “thrombotic microangiopathies” (TMA) refers to clinical disorders characterized by the shared features of microangiopathic hemolytic anemia, thrombocytopenia, and microvascular thrombotic lesions. The period since 2002 has witnessed significant advances in the understanding of the entities included under TMA, particularly their genetics and pathophysiologic etiologies. Historically, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) were the major clinical conditions included in a discussion of TMA. The two share certain clinical and pathologic features, and for a number of years it was recommended that the two be included within the combined clinical classification of “TTP-HUS”. However, the disorders appear to be different entities, with TTP associated with a severe deficiency of the von Willebrand factor cleaving protease ADAMTS-13, and familial and some sporadic cases of HUS associated with a deficiency of complement regulatory proteins. The current understanding of these two disorders is summarized in the following article by Margaret Schneider.

Medical textbooks have historically considered arterial and venous thromboembolic disease as distinct entities, with different pathophysiologic bases, unique risk factors, and distinct therapeutic approaches. However, a number of recent studies suggest that the classic distinction between the two may be artificial. Dorothy Adcock summarizes these findings and suggests that the diagnostic and treatment protocols for these patients may need to change.

The introduction of the laboratory assay for high-sensitivity C-reactive protein (hs-CRP) in the hemostasis laboratory evaluation of patients at risk for cardiovascular disease was an early recognition of the interaction between coagulation and inflammation. These two physiologic systems actually interact at multiple levels, and the clinical hemostasis laboratory can expect additional “inflammation” assays to be added to the coagulation test menu. The physiologic basis of their interactions is summarized in “Cross Talk Between the Inflammation and Coagulation Systems”.

J Lynne Williams PhD CLS(NCA) is the Focus: New Directions in Homeostasis and Coagulation guest editor.
Detection of the proteolytic enzyme ADAMTS-13 may be used to differentiate between the forms of thrombotic microangiopathy (TMA), thrombotic thrombocytopenic purpura (TTP) and hemolytic-uremic syndrome (HUS).

**ABBREVIATIONS:** ADAMTS = a disintegrin-like and metalloprotease domain with thrombospondin type motifs; HUS = hemolytic-uremic syndrome; LD = lactate dehydrogenase; MAHA = microangiopathic hemolytic anemia; PT = prothrombin times; PTT = partial thromboplastin times; TMA = thrombotic microangiopathy; TT = thrombin times; TTP = thrombotic thrombocytopenic purpura; ULVWF = ultra-large VWF; VTEC = verotoxin-producing E. coli; VWF = Von Willebrand factor; VWF-cp = VWF-cleaving protease.

**INDEX TERMS:** ADAMTS-13; hemolytic-uremic syndrome; microangiopathic hemolytic anemia; schistocytes; thrombotic microangiopathy; thrombotic thrombocytopenic purpura.

**LEARNING OBJECTIVES**
1. Compare and contrast the clinical symptoms and etiology of TTP and HUS.
2. Name the enzyme responsible for cleaving ultra large von Willebrand factor molecules.
3. Describe the clinical manifestations of a deficiency of this enzyme.
4. Predict the most appropriate course of treatment for a patient with this enzyme deficiency.

Thrombotic microangiopathy (TMA) is a form of systemic thrombosis with microangiopathic hemolytic anemia (MAHA), thrombocytopenia, and microthrombi formation within arterioles and capillaries. One third of patients have hemoglobin values below 6 g/dL. MAHA is a form of anemia characterized by elevated reticulocyte count and serum lactate dehydrogenase (LD), decreased serum haptoglobin, and presence of schistocytes (fragmented erythrocytes) on the peripheral blood film. Most patients have normal prothrombin times (PT), activated partial thromboplastin times (PTT), and thrombin times (TT). When left untreated, patients die from systemic microvascular thrombosis causing cerebral and myocardial infarctions and renal failure. In the case reported by Moschcowitz in 1924, a 16-year-old female died of a form of TMA that included MAHA, petechiae, hemiparesis (partial paralysis) and fever. Autopsy findings included hyaline thrombi in the terminal arterioles and capillaries of the heart and kidney.

When a patient presents with symptoms of gastroenteritis including abdominal pain and diarrhea, a significantly decreased platelet count, and schistocytes on the peripheral smear but normal coagulation studies, TMA should be strongly considered. Prompt recognition is important because this condition must be classified as either TTP or HUS. Both disorders present with thrombocytopenia and MAHA. Timely treatment with plasma-exchange is very effective for individuals with TTP while HUS does not respond to the same treatment. The majority of patients with HUS recover normal renal function with only supportive care, while if left untreated (and in the era before effective treatment) TTP mortality exceeds 90%.
HEMOLYTIC UREMIC SYNDROME

HUS and TTP overlap clinically as patients present with similar symptoms and the laboratory finding of MAHA (Table 1). Differentiation is important due to the unique treatment for each. HUS is a TMA that mainly affects the kidney and may cause oliguric or anuric renal failure. Diarrhea-positive or ‘typical’ HUS (also referred to as D-HUS) is associated with fever, bloody diarrhea and infection by verotoxin-producing E. coli (VTEC). *Escherichia coli* O157:H7 accounts for approximately 80% of cases, but D-HUS can be caused by other toxin-bearing *E. coli* serotypes or by *Shigella dysenteriae* type 1. Approximately 15% of patients with diarrhea caused by verotoxin-producing *E. coli* develop D-HUS. The exact pathogenesis of D-HUS remains undefined, but is thought to be associated with toxin-related renal vascular damage. Ninety-five percent of all cases of D-HUS are found in children, with occasional cases in adults.

The remaining HUS cases are referred to as diarrhea-negative or atypical HUS (D-HUS), and are not associated with VTEC infections. D-HUS is usually due to an inherited abnormality of the complement regulatory system that is familial, chronic, and relapsing. Patients demonstrate persistently low levels of complement, often caused by a homozygous deficiency of complement factor H. Deficiencies of other complement regulatory proteins, membrane cofactor protein (MCP) or factor I, have also been reported. Although most cases of HUS present with thrombocytopenia, platelet counts are generally not as decreased as those seen in TTP. Neurologic symptoms are also less common and less severe; seizures, coma, or stroke occur in approximately ten percent of patients. Kidney microthrombi in HUS often result in renal insufficiency.

The goal of initial management of HUS patients is to maintain renal perfusion with intravenous fluids while avoiding fluid overload. Approximately 50%-60% of patients with renal insufficiency and a diminishing urine output progress to oliguric renal failure, requiring dialysis. However approximately 90% of children with D-HUS survive with supportive care. No beneficial effect of plasma therapy has been proven for VTEC-associated HUS, and the role of antibacterial therapy for enteritis associated with the infection is controversial. Antibiotics may actually increase the risk of HUS in patients with VTEC infections. Approximately one-third of patients experience renal impairment for many years following the initial HUS event.

THROMBOTIC THROMBOCYTOPENIA PURPURA

The recognition and diagnosis of TTP can be challenging due to the variety of clinical presentations and lack of specific diagnostic criteria. Historically, TTP was defined by the “classic” pentad of severe thrombocytopenia, hemolytic anemia with numerous schistocytes, neurologic findings, renal damage, and fever. However MAHA, schistocytes, and thrombocytopenia are the only consistent features, and these also occur in HUS and disseminated intravascular coagulation. Other TTP laboratory features include increases in reticulocytes and nucleated red blood cells on the peripheral smear. Patients may present with varying degrees of abdominal pain, nausea, vomiting and weakness. Neurologic symptoms may include headache, visual disturbances, vertigo, confusion, lethargy, coma, seizures, aphasia or hemiparesis; these are seen in 50% to 90% of TTP cases. These symptoms may even occur in the weeks preceding physician

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**Table 1. Comparison of TTP and HUS**

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<th>Feature</th>
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<th>HUS</th>
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<td>Yes</td>
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<tr>
<td>O157:H7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal failure</td>
<td>Uncommon</td>
<td>Common</td>
</tr>
<tr>
<td>Neurologic symptoms</td>
<td>Common</td>
<td>Uncommon</td>
</tr>
<tr>
<td>Organ involvement</td>
<td>Multiple organs</td>
<td>Limited to kidney</td>
</tr>
<tr>
<td>VWF multimer</td>
<td>Ultra-large forms present</td>
<td>Smaller multimers predominate</td>
</tr>
<tr>
<td>ADAMTS-13 activity</td>
<td>Deficient</td>
<td>Normal</td>
</tr>
</tbody>
</table>
evaluation. Although TTP is often considered an acute clinical event, one-fourth of patients experience symptoms several weeks before diagnosis. Systemic microvascular thrombosis can affect any organ. Renal involvement occurs in approximately 50% of patients, however acute renal failure occurs in less than 10%. Fever is uncommon, and TTP typically occurs in adult females over 40 years old. The female-to-male ratio averages approximately 2:1, but this ratio approaches equality after age 60. Obesity and African ancestry are also associated with an increased risk, and the relative risk ratio of “blacks” to “non-blacks” is approximately 9:1.

Von Willebrand Factor and TTP
Von Willebrand factor (VWF) is a multimeric glycoprotein synthesized by endothelial cells and megakaryocytes that plays an important role in primary hemostasis by bridging platelets with exposed vascular endothelium under conditions of high shear rates. When secreted from storage organelles of activated endothelial cells, VWF appears in the form of ultra-large VWF multimers (ULVWF). ULVWF multimers are more effective in inducing platelet adhesion and aggregation than typical large VWF forms that circulate in normal plasma. Patients with chronic, relapsing TTP contain ULVWF forms in their plasma. In 1982 Moake and others suggested that the presence of ULVWF multimers might be due to either excessive release of VWF from endothelial cells or impaired degradation of the highly multimeric forms of VWF by a ‘depolymerase’. Furlan called this activity VWF-cleaving protease (VWF-cp). In addition, Furlan studied two brothers with chronic, relapsing TTP and detected ULVWF multimers with no VWF-cp activity in their plasma. Furlan further found ULVWF multimers to be absent from plasma from their asymptomatic parents who had 50% VWF-cp activity. No VWF-cp inhibitor was detected.

ADAMTS-13
In flowing blood, shear forces stretch VWF multimers and expose an A2 domain cleavage site for VWF-cp. Cleavage divides VWF into smaller subunits and releases adhered platelets back to the circulation. Insufficient cleavage results in thrombosis, and excessive cleavage may cause bleeding.

Independent studies in 1996 by Furlan and Tsai identified VWF-cp as 1 of 19 members of the ADAMTS (A Disintegrin-like And Metalloprotease domain with ThromboSpondin type motifs) family of metalloproteases, designated ADAMTS-13. ADAMTS-13 digests ULVWF multimers as
they are synthesized and secreted by endothelial cells. The enzyme cleaves the bond between tyrosine at position 842 and the methionine residue at position 843 within the A2 domain.13 In the circulation, ADAMTS-13 docks to the surface of endothelial cells where ULVWF multimers are present and cleaves them to smaller, normal VWF units (Figure 1). When ADAMTS-13 is significantly reduced or absent, the resulting ULVWF multimers adhere to platelets, causing platelet agglutination and the disseminated platelet thrombi characteristic of TTP (Figure 2).6

The discovery and identification of ADAMTS-13 explained the etiology of many familial and acquired cases of TTP. The gene encoding ADAMTS-13 has been mapped to chromosome 9q34. The protein is produced in the liver as a zymogen and becomes activated upon propeptide cleavage. In 2002 Dong and others demonstrated that newly released ULVWF forms long string-like structures on the surface of stimulated endothelium, capable of supporting platelet adhesion and agglutination. They are rapidly cleaved in vitro in the presence of normal plasma or partially purified ADAMTS-13 but not in the presence of plasma from patients with TTP.14 Failure to cleave ULVWF multimers has dangerous clinical consequences as the aggregated platelets may occlude small vessels, dislodge under high shear stress, and obstruct smaller vessels downstream leading to tissue ischemia and infarction, the classic clinical presentation of TTP. Additionally, platelets may be activated on adhesion to the ULVWF multimers and release substances with the potential to activate or damage endothelial cells.

**DIAGNOSIS AND CLINICAL PRESENTATION**

When a patient presents with thrombocytopenia, anemia, abdominal pain, nausea, vomiting, and weakness, the diagnosis of TTP should be considered. Gastroenteritis, sepsis, HUS, or DIC need to be ruled out. Differential diagnosis requires laboratory evaluation. Coagulation studies, including the PT, PTT, TT, and fibrinogen concentration, are normal in TTP. Key diagnostic clues are severely decreased platelet counts (as low as 10 x 10^9/L), schistocytes and nucleated red blood cells on the peripheral film, an increased reticulocyte count, and a negative direct Coombs’ test.

TTP may be hereditary or acquired. Schulman and Upshaw described a form of autosomal recessive TTP, now called the Upshaw-Schulman syndrome.15,16 In the late 1950s, Schulman investigated a patient with chronic thrombocytopenia, petechiae, and ecchymoses. He concluded the patient lacked a factor present in normal plasma, as a rise in platelet count ensued when plasma transfusion was used as therapy.15 Hereditary mutations of the ADAMTS-13 protein are now known to be the cause of the Upshaw-Schulman syndrome.17 Gene mapping studies have discovered multiple mutations in almost all structural domains of the molecule. Patients experience early childhood onset of symptoms, and the condition may be fatal within the first few weeks of life if untreated. These patients lack ADAMTS-13 protease, typically having protein levels at zero percent to ten percent of normal. Upshaw-Schulman syndrome is quite rare.

Acquired or sporadic TTP has been found in a different clinical population. Also termed idiopathic TTP, patients may present with the classic pentad of symptoms (MAHA, thrombocytopenia, neurologic symptoms, fever, and renal dysfunction).3,7 However, only approximately 40% of patients develop the complete pentad; 75% present with the triad of MAHA, neurologic symptoms, and thrombocytopenic purpura. The reported incidence of fever and renal dysfunction has diminished, most likely due to earlier diagnosis.7 Mortality exceeded 90% prior to the recognition that blood or plasma infusions improved the outcome of TTP; prompt treatment with plasma exchange therapy has increased long-term survival to 78-90%.3 Acquired TTP is caused by autoantibodies, mainly IgG, inhibiting ADAMTS-13 activity.18,19 Acquired TTP may be secondary to autoimmune diseases, cancer and chemotherapy, ticlopidine, clopidogrel, cyclosporin, mitomycin C, allogeneic bone marrow or solid organ transplantation, viral infections (HIV), and pregnancy. Most cases of acquired TTP occur once, with occasional patients demonstrating a relapse or secondary episode.7

Once the tentative diagnosis of TTP has been made, suitable treatment should begin without delay. In most institutions, this treatment includes daily plasmapheresis for seven days or until the platelet count returns to normal. The effectiveness of plasma exchange therapy has been attributed to both the removal of ADAMTS-13 autoantibodies and the replacement of ADAMTS-13 activity. If plasmapheresis is discontinued prematurely, the condition may reoccur rapidly.

Laboratory testing for ADAMTS-13 is useful, however not required, for diagnosis or to begin treatment. Most institutions send plasma samples to a reference laboratory for analysis. A technique developed by Kokame and others uses a Fluorescence Resonance Energy Transfer (FRET) assay.
whereby a fluorescent signal is detected when a synthetic substrate (FRET-VWF73) is cleaved by ADAMTS13. In addition, antibody or inhibitor activity against the protease is measured by a Bethesda-type method similar to that used to measure antibodies to factor VIII. The inhibitor activity is determined by measuring the ability of heat treated patient plasma to inhibit ADAMTS13 present in normal pooled plasma. Both ADAMTS-13 activity and inhibitor levels should be reported to give a complete diagnostic picture.

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Is There a Genetic Relationship Between Arterial and Venous Thrombosis?

DOROTHY M ADCOCK

ABBREVIATIONS: ASVD = atherosclerotic vascular disease; DVT = deep venous thrombosis; GAIT = Genetic Susceptibility to Thrombosis; PE = pulmonary embolus; VTE = venous thromboembolic event.

INDEX TERMS: acute coronary syndrome; atherosclerotic vascular disease; deep vein thrombosis; lipids; myocardial infarction; pulmonary embolism; stroke; venous thromboembolic disease.


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LEARNING OBJECTIVES
1. Correlate the clinical pathologic manifestations with the type of thrombosis (arterial vs venous).
2. Compare and contrast the most important risk factors, the nature of the clot, and the target for therapeutic prevention and treatment for arterial and venous clot formation.
3. Summarize the data suggesting a link between arterial and venous thrombosis.

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Thromboembolic disease is the leading cause of morbidity and mortality in the developed world. Arterial thrombosis is the most common underlying cause of acute myocardial infarction, non-hemorrhagic cerebrovascular accidents, and peripheral vascular disease. Pathological manifestations of venous thrombosis largely include deep venous thrombosis (DVT) and/or pulmonary embolus (PE). While arterial thromboembolic events are the foremost cause of death and disability, venous disease also plays an important role. DVT affects approximately two million Americans annually while PE is the most common cause of preventable hospital death accounting for 60,000 deaths in the United States annually.

Medical textbooks and epidemiological studies characteristically consider arterial and venous thromboembolic disease as distinct entities, each with their own pathophysiological basis, unique risk factors, and distinct therapeutic regimens. Arterial clots typically occur in an injured vessel and the most common cause of vascular damage in the arterial system is atherosclerotic vascular disease (ASVD). The risk factors for arterial thrombosis are therefore considered the same as those for ASVD. Arterial clots occur in a high flow, high shear environment and these clots, also called white clots, are rich in platelets. Prevention and treatment of arterial thrombosis is often aimed at platelet inhibition. While vascular injury can promote the formation of venous clots, stasis and changes in blood composition (thrombophilia) are the most important risk factors for venous clot development. Venous clots occur in a low flow system. They are rich in fibrin that is enmeshed with red blood cells and are referred to as red clots. Inhibition of fibrin formation is the mainstay of prevention and treatment of venous thrombosis. It is often reported that the risk factors for arterial and venous thrombosis largely differ.

Is this distinction between arterial and venous thrombosis artificial? Is it a by-product of the fact that these entities often occur in different clinical settings and that they are treated differently and by different physician specialists? Have research and epidemiological studies, in evaluating arterial and venous disease as separate entities, perpetuated this segregated approach? Is it possible or perhaps likely that arterial and venous thromboembolic events actually represent different presentations of the same disease? While it is appreciated that...
one may provoke the other as either can activate the hemostatic system, is the relationship between these entities in fact, more intimate? One important consideration supporting a close link is the well appreciated concept that both arterial and venous thrombosis are complex multi-factorial traits in which multiple genetic and acquired risk factors interact to determine risk.\textsuperscript{4,6,7} Do the risk factors for arterial and venous thrombosis share more in common than previously realized? If so, are there common genetic and acquired components? Indeed, recent studies, including a select few discussed below, have demonstrated a close association between these entities at a variety of levels. Specifically it has been shown that 1) arterial and venous thrombosis share common risk factors, 2) individuals that suffer idiopathic VTE are at a markedly increased risk of suffering a significant cardiovascular event, 3) individuals that suffer idiopathic VTE have an increased incidence of atherosclerotic vascular disease, and 4) those that suffer idiopathic VTE have a significantly higher incidence of metabolic syndrome.

The risk factors reported to be common to both arterial and venous thrombosis and that represent significant hazard for the development of each entity include increasing age and weight, smoking, exposure to estrogen, and the presence of diabetes. It has also been shown that high HDL cholesterol levels are associated with a decreased risk of venous thrombosis while elevated triglyceride and/or total cholesterol levels convey an increased risk.\textsuperscript{8,9} Other risk factors reported to be common to both arterial and venous thrombosis include the presence of antiphospholipid antibodies, dysfibrinogenemia, hyperhomocysteinemia, and elevated levels of fibrinogen, lipoprotein (a) and factor VIII.\textsuperscript{10,11}

A number of studies published recently have reported a strong link between arterial and venous thromboembolic disease. These studies have shown that patients who suffer an idiopathic venous thromboembolic event (VTE) are more likely to experience a significant arterial event than either the general population or those who suffered a provoked venous thrombosis.\textsuperscript{12} In a 2005 study published in the European Heart Journal, of those who suffered their first idiopathic PE, 11% had a symptomatic arterial event with 12% mortality while those with a PE secondary to a known provocateur suffered a 4% rate of symptomatic arterial events with 4% mortality. Another study published in 2006 reported that 11% of those who suffered an idiopathic VTE had a symptomatic arterial event with 8% mortality compared to 4% in the secondary VTE group with a 3% mortality rate. Likewise, Prandoni and others reported a 60% higher risk of suffering arterial events in those with idiopathic VTE than the cohort with VTE secondary to well-established risk factors after adjusting for age, hypertension, smoking, gender, and the presence of diabetes.\textsuperscript{13} Cardiovascular events therefore represent a major cause of morbidity and mortality in those individuals also at risk for idiopathic PE. This link supports a strong connection between arterial and venous thrombosis.\textsuperscript{12} Given these reports, idiopathic PE can be considered an independent risk factor for cardiovascular disease.

In a recent study reported in the New England Journal of Medicine, patients with idiopathic VTE who were asymptomatic for atherosclerosis were studied using carotid ultrasound.\textsuperscript{14} Carotid plaques are considered a reliable indicator of atherosclerosis elsewhere in the circulation, in subjects without symptomatic atherosclerosis. Forty-seven percent of those individuals with idiopathic VTE had carotid plaque compared to twenty-seven percent with secondary DVT and thirty-two percent of controls.\textsuperscript{14} After noting this association between spontaneous VTE and atherosclerotic disease, the authors hypothesized that either one induces the other or else they share common risk factors.

There is an apparent association between metabolic syndrome, characterized by insulin resistance or glucose intolerance, hypertension, atherogenic dyslipidemia, central obesity, and the development of idiopathic VTE.\textsuperscript{15} It is well-established that those with metabolic syndrome are at significantly increased risk for the development of cardiovascular events. These individuals are also at risk for suffering spontaneous VTE. Ageno demonstrated that patients with idiopathic VTE had a higher incidence (51%) of metabolic syndrome than those without VTE (35%) or those that suffered VTE with known provocateur.\textsuperscript{15} Should individuals with metabolic syndrome be more likely to receive thromboprophyaxis in situations of increased thrombotic risk than those without metabolic syndrome?

One of the strongest pieces of evidence in favor of a link between arterial and venous thrombosis is the Genetic Susceptibility to Thrombosis (GAIT) study.\textsuperscript{16} This family-based study of the genetics of thrombosis in a Spanish population was initiated to determine the heritability of thrombosis. Three hundred and twenty-eight individuals in thirty-two extended pedigrees were evaluated using a novel computer assisted adaptation of a multivariate threshold model. The authors concluded that more than 60% of the variation in susceptibility to common thrombosis is attributable to genetic factors. What makes this study unusual is that both
venous and arterial thromboembolic events were included in the analysis. When venous and arterial thrombosis were jointly analyzed as two distinct traits, the genetic correlation between these entities was not significantly different from one, and this strongly suggests that arterial and venous thromboses are highly genetically correlated. That is, many of the same genes are involved in the pathogenesis of arterial and venous disease.

The recent studies cited suggest that arterial and venous thrombosis represent different manifestations of the same disease and that the underlying process is driven by a common set of genes. The implications of these findings are significant with potential impact on many different areas of medicine from basic research to the various patient treatment regimens in current use. Transgenic and knock-out murine models evaluating thrombotic risk should evaluate risk of both arterial and venous disease, as should large family studies using genome wide linkage analysis. On a more practical note, the close link demonstrated between arterial and venous thrombosis suggests that patients who suffer idiopathic VTE should be closely evaluated for risk of all cardiovascular events and that individuals with atherosclerotic vascular disease should be monitored or in some circumstances provided prophylaxis for VTE. Future trials are needed to determine whether the long-term management of VTE should include prophylaxis against arterial cardiovascular events.

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REFERENCES
Cross Talk Between the Inflammation and Coagulation Systems

J LYNNE WILLIAMS

ABBRREVATIONS: APC = activated protein C; AT = antithrombin; C4bBP = C4b binding protein; DIC = disseminated intravascular coagulation; EC = endothelial cells; EPCR = endothelial cell protein C receptor; GAG = glycosaminoglycans; PAF = platelet activating factor; PAI-1 = plasminogen activator inhibitor-1; PARs= protease activated receptor; PC = protein C; PDGF = platelet derived growth factor; TAFI = thrombin activated fibrinolysis inhibitor; TFPI = tissue factor pathway inhibitor; TM = thrombomodulin; TNF = tumor necrosis factor; ULVWF = ultra-large multimers of von Willebrand factor.

INDEX TERMS: coagulation; cytokines; endothelial cells; hemostasis; inflammation; leukocytes.

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LEARNING OBJECTIVES

1. Identify the major components (cellular and inflammatory mediators) of inflammation.
2. Describe the functions of the inflammatory response.
3. List the major inflammatory cytokines.
4. Identify the significant effects of the inflammatory mediators on the coagulation and fibrinolytic systems.
5. Identify the significant effects of the coagulation and fibrinolytic systems on inflammation.

Previously, blood coagulation and inflammation were thought to be completely different physiologic processes. It is now recognized, however, that these two systems are interrelated as part of the host defense mechanism. In invertebrates, the functions of clotting and inflammation are mediated by a single cell system, the hemocyte. In vertebrates, clotting and inflammation have diverged into the specialized functions of the platelets, phagocytic cells (neutrophils and macrophages), and several plasma protein systems (procoagulant proteins, complement proteins, proteins of the kinin system).

Several observations suggest an ongoing interaction between these two systems. There is significant structural homology between the proteins of the complement and coagulation cascades. Both cascades utilize serine proteases and are activated through a series of proteolytic cleavage reactions. Animals subjected to experimental sepsis have multi-organ failure associated with activation of blood coagulation (mediated primarily by inflammatory mechanisms). Inhibition of coagulation factor Xa activity inhibits fibrin formation but does not reduce mortality in this model system. However, inhibition of tissue factor/factor VIIa activity or infusion of activated protein C not only reduces fibrin formation, but also significantly reduces mortality. Thus, there are interactions between the proteins of these two systems that go beyond simply the activation of fibrin formation.

Both coagulation and inflammation are essential parts of the host defensive response. These pathways have several connecting points through which they interact. Importantly, the interaction between coagulation and inflammation is bidirectional: both inflammation-induced coagulation as well as coagulation-induced inflammation occur.

THE INFLAMMATORY RESPONSE

The inflammatory response refers to the biochemical and cellular processes that occur in vascularized tissue in response
to injury or infection. It encompasses a complex series of events fundamental to the ability of the human body to protect itself against injurious and infectious agents. The inflammatory response requires the coordinated interaction of 1) local resident cells in the tissues (macrophages, mast cells, fibroblasts); 2) vascular endothelial cells; 3) plasma mediator systems; 4) leukocytes.

The functions of the inflammatory response are: to neutralize or eliminate the offending agent (or to destroy the necrotic tissue); if this cannot be accomplished, the inflammatory response attempts to wall off and confine these agents to limit their effects on the host; to stimulate and enhance the adaptive immune response; and to promote healing.

The classic macroscopic hallmarks of inflammation have been recognized for more than 5000 years and include redness, swelling, heat, and pain. At the microscopic level, these can be explained by the dilation of blood vessels, which increases blood flow into the area, an increase in vascular permeability resulting in the outward leakage of plasma from the vessels into the tissues, and the adherence and emigration of leukocytes through the vascular wall into the surrounding tissues at the site of injury.

The major components of the inflammatory response are listed in Table 1. The process is regulated by a number of inflammatory mediator systems, listed in Table 2. Inflammatory mediators include endogenous (from cells or plasma) as well as exogenous (from bacteria or other pathogens) substances. As can be seen from the table, the list includes a number of components from the coagulation/hemostatic system.

Thus the blood vessels and blood, in addition to being the transport mechanism for the distribution of nutrients and oxygen throughout the body (and the removal of metabolic waste products), are also the essential components of the body’s natural immunity (defense) against foreign pathogens and defense against tissue injury. Following injury or infection, an inflammatory response is activated, the integrity of the endothelium is compromised, and phagocytic cells are activated. The endothelium, which is normally anticoagulant and antithrombotic in the resting state, changes to a procoagulant surface, and the balance of the hemostatic system shifts towards hypercoagulability.

**INFLAMMATION AND COAGULATION**

It has long been recognized that systemic inflammation is associated with hypercoagulability, as evidenced by the common occurrence of disseminated intravascular coagulation (DIC) in severe sepsis. More recently, the molecular basis of this association has been determined. Most of the hypercoagulable effects of inflammation are mediated via the inflammatory cytokines, including IL-1, IL-6 and tumor necrosis factor (TNF).

The hypercoagulability associated with systemic inflammation is due primarily to an increase in procoagulant functions, an inhibition of fibrinolysis, and a downregulation of the three major physiologic anticoagulant systems (activated protein C/APC, antithrombin/AT, tissue factor pathway inhibitor/TFPI, Figure 1):

1. *Decreased activity of the protein C (PC) anticoagulant system* (Figure 2). This is due to decreased endothelial cell expression of thrombomodulin (TM), triggered by IL-6, IL-1 and TNF. TM binds thrombin to activate PC to activated protein C (APC). These cytokines also decrease endothelial cell expression of the endothelial cell PC receptor (EPCR), which binds and localizes PC, facilitating its activation by TM/thrombin. In addition, they trigger the hepatic “acute phase response” resulting in increased synthesis and secretion of several acute phase proteins, including C4b binding protein (C4bBP). C4bBP binds plasma protein S, reducing the quantity of “free” protein S. Free protein S is a cofactor for the APC degradation of the procoagulant proteins activated Factor Va and Factor Vlla.

2. *Decreased activity of other coagulation inhibitors* (Figure 2). Both tissue factor pathway inhibitor (TFPI) and antithrombin (AT) are “negative acute phase response proteins” (meaning their synthesis and secretion is reduced during the acute phase response). Inflammatory cytokines decrease EC expression of glycosaminoglycans (GAG), the endogenous cofactor for activation of AT activity.

<table>
<thead>
<tr>
<th>Table 1. Components of the inflammatory response</th>
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<tbody>
<tr>
<td><strong>Cells</strong></td>
</tr>
<tr>
<td>PMNs (neutrophils)</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
</tr>
<tr>
<td>Eosinophils</td>
</tr>
<tr>
<td>Basophils/mast cells</td>
</tr>
<tr>
<td>Platelets</td>
</tr>
</tbody>
</table>
3. **Increased procoagulant activity of the endothelium.** Inflammatory cytokines upregulate endothelial expression of tissue factor, the procoagulant protein required for factor VIIa activity, and down regulate endothelial expression of heparin sulfate/glycosaminoglycans, the cofactors for the anticoagulant activity of antithrombin.\(^\text{15}\)

4. **Increase in plasma procoagulants.** Plasma fibrinogen levels rise, as fibrinogen is another acute phase protein.\(^\text{16}\) Inflammatory mediators induce the endothelial release of ultra-large multimers of von Willebrand factor (ULVWF)—which are hemostatically more effective in inducing platelet activation responses.\(^\text{17}\) They also inhibit the cleavage of ULVWF by ADAMTS13. Inflammatory mediators upregulate TF expression on circulating monocytes, shedding of monocyte-derived microparticles, and initiating systemic coagulation\(^2\) (Figure 3).

5. **Decreased activity of the fibrinolytic system.** Inflammatory cytokines stimulate endothelial cells to increase production of plasminogen activator inhibitor-1 (PAI-1).\(^\text{18,5}\)

6. **Increased platelet count and platelet reactivity.** IL-6 promotes platelet production and is also associated with an increased platelet reactivity (as the newly formed platelets are activated at lower concentrations of agonists).\(^\text{19}\)

### INFLUENCE OF COAGULATION ON INFLAMMATION

Plasma procoagulant enzymes stimulate and augment the inflammatory response. Coagulation inhibitor systems have anti-inflammatory activities in addition to their anti-coagulant functions:

1. **Thrombin, factors Xa and VIIa**, in addition to their roles in activating coagulation protein zymogens, can interact with specific cell receptors and activate intracellular signaling pathways that mediate inflammatory responses. These enzymes bind protease activated receptors (PARs) on cell membranes and induce a cellular response (Figure 4).\(^2,20,21\)

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**Table 2. Inflammatory mediator systems**

<table>
<thead>
<tr>
<th>Mediator system</th>
<th>Major actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasoactive amines: Histamine, serotonin</td>
<td>Induction of adhesion, ↑P-, E-selectin, Vasodilation, ↑vascular permeability</td>
</tr>
<tr>
<td>Vasoactive peptides: Kinins (bradykinin)</td>
<td>Vasodilation, ↑vascular permeability, Endothelial contraction; pain</td>
</tr>
<tr>
<td>C3a, C5a</td>
<td>↑vascular permeability, Chemotaxis; activation of mast cells</td>
</tr>
<tr>
<td>Other complement components: C3b, C56789</td>
<td>Opsonization (assist phagocytosis), Cytolysis</td>
</tr>
<tr>
<td>Coagulation/fibrinolytic: Fibrinopeptides A, B (FPA,FPB), Fibrin degradation products (FDP), Plasmin</td>
<td>↑vascular permeability; chemotactic, ↑vascular permeability; chemotactic, Generates FDPs; activates C3, factor XIIa, prekallikrein, releases kinins</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Releases FPA, FPB; ↑P-, E-selectin; Activate endothelial cells, platelets; Chemotactic; induce cytokine release</td>
</tr>
<tr>
<td>Phospholipids: Prostaglandins, leukotrienes</td>
<td>Vasodilation; ↑vascular permeability; Chemotaxis</td>
</tr>
<tr>
<td>Platelet activating factor (PAF)</td>
<td>↑vascular permeability; cell activation</td>
</tr>
<tr>
<td>Reactive oxygen intermediates: O(_2), H(_2)O(_2), HO(-)</td>
<td>Tissue damage (cytolysis); activation of complement; generate chemotactic lipids</td>
</tr>
<tr>
<td>Lysosomal granule contents</td>
<td>Tissue damage (proteolysis); matrix degradation; generate oxidant reactions</td>
</tr>
<tr>
<td>Cytokines/chemokines</td>
<td>Cell activation; induction of adhesion, chemotaxis, fever, acute-phase response</td>
</tr>
</tbody>
</table>
2. Thrombin stimulates endothelial cells (EC), promoting vasoconstriction and increased vascular permeability; promotes adhesion and transendothelial migration of leukocytes; stimulates EC and fibroblasts to produce inflammatory cytokines; activates platelets, inducing the release of platelet-derived inflammatory mediators.\(^{22,23}\)

3. Factors VIIa and Xa promote leukocyte adhesion and migration and the synthesis and secretion of inflammatory cytokines.\(^{22,24}\)

4. Activated protein C (APC) can bind to a PAR, and generate signals that inhibit the inflammatory response (Figure 5).\(^{22}\)

5. AT, APC, and TFPI all interact with inflammatory cells and decrease the production of inflammatory mediators, and leukocyte activation in inflammation (Figure 5).\(^{2,5}\)

6. Thrombin activated fibrinolysis inhibitor (TAFI), in addition to inhibiting fibrinolysis, inactivates proinflammatory mediators including C3a, C5a, and bradykinin (Figure 5).\(^{2,5,25}\)

7. Platelets are a rich source of inflammatory mediators; when activated, they secrete a number of factors that promote vascular reactions, leukocyte chemotaxis, endothelial cell activation, and other aspects of the inflammatory response including serotonin, platelet activating factor (PAF), and platelet derived growth factor (PDGF).\(^{1,26}\)

CLINICAL IMPLICATIONS OF “CROSS TALK” BETWEEN INFLAMMATION AND COAGULATION

The processes of coagulation, thrombosis, and inflammation do not happen in isolation. There is a clear interaction...
between all three, in which thrombosis and coagulation can act as triggers for inflammation, and severe or systemic inflammatory responses can trigger coagulation.

An early outcome of the understanding of the interaction between these processes was the use of the laboratory test, high sensitivity C-reactive protein (hsCRP), as a marker for atherosclerosis and coronary artery disease.\(^ {27,28}\) It is now recognized that an elevated hsCRP may be an early, pre-clinical symptom indication of atherosclerotic inflammatory disease and impending acute thrombotic events. Similarly, studies are underway to evaluate the usefulness of serum IL-6 assays as an indication of the same.

A better understanding of the inflammation/coagulation interface has prompted ongoing research on the interaction of coagulation and inflammation in a variety of disease processes, including inflammatory bowel disease, rheumatoid arthritis, and malignancy. An intriguing study suggesting the importance of the contribution of inflammation to atherothrombosis reported that patients with rheumatoid arthritis exhibited increased risk of myocardial infarction.\(^ {29}\) Additional clinical implications of the interaction of these two systems in other disease processes is likely.

An important outcome of the recognition that inflammation and coagulation are intricately related processes is the use of natural anticoagulants as a treatment in acute inflammatory diseases, such as severe sepsis. Activated protein C has been shown to significantly decrease and morbidity and mortality associated with severe sepsis in an animal model,\(^9\) as well as in early human clinical trials.\(^ {30}\) As our understanding of the molecular mechanisms underlying the close relationship between these two systems progresses, it is likely that new potential targets for therapies will be evaluated, capable of modulating excessive activation of both systems simultaneously.

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**Figure 4. Coagulation proteases and inflammation**

Activated coagulation proteases (factor IIa or thrombin, factor VIIa, factor Xa) not only interact with coagulation protein zymogens, but also with specific cell receptors (PARs) to induce signaling pathways that mediate inflammatory responses. PARs are protease activated receptors (of which there are four types, PAR 1 to 4) which, upon cleavage by the coagulation protease, initiates transmembrane signaling, and the induction of genes necessary for the inflammatory response (e.g. synthesis of inflammatory cytokines, cell adhesion molecules, activation of inflammatory responses in macrophages).

TF = Tissue factor; PAR = Protease activated receptor; VIIa = activated factor VII; Xa = activated Factor X; IIa = thrombin (activated factor II)

**Figure 5. Anti-inflammatory actions of anticoagulant proteins**

The natural anticoagulant proteins also have anti-inflammatory actions. These include antithrombin, activated TAFI, activated Protein C.

TM = thrombomodulin; F-IIa = activated factor II or thrombin; TAFI = Thrombin activatable fibrinolysis inhibitor; AT = Antithrombin; APC = Activated protein C; EPCR = Endothelial cell protein C receptor; PAR-1 = Protease activated receptor-1; F Va = activated factor V; F VIIa = activated factor VIII
FOCUS: NEW DIRECTIONS IN HEMOSTASIS AND COAGULATION

REFERENCES


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Continuing Education Questions

FALL 2007

To receive 2.0 contact hours of intermediate level, P.A.C.E.* credit for the Focus: New Directions in Hemostasis and Coagulation questions, insert your answers in the appropriate spots on the continuing education registration form that follows, then mail a photocopy of the form as directed.

LEARNING OBJECTIVES
Upon completion of this section, the reader will be able to:
1. compare and contrast the clinical symptoms and etiology of TTP and HUS.
2. name the enzyme responsible for cleaving ultra large von Willebrand factor molecules.
3. describe the clinical manifestations of a deficiency of this enzyme.
4. predict the most appropriate course of treatment for a patient with this enzyme deficiency.
5. correlate the clinical pathologic manifestations with the type of thrombosis (arterial vs venous).
6. compare and contrast the most important risk factors, the nature of the clot, and the target for therapeutic prevention and treatment for arterial and venous clot formation.
7. summarize the data suggesting a link between arterial and venous thrombosis.
8. identify the major components (cellular and inflammatory mediators) of inflammation.
9. describe the functions of the inflammatory response.
10. list the major inflammatory cytokines.
11. identify the significant effects of the inflammatory mediators on the coagulation and fibrinolytic systems.
12. identify the significant effects of the coagulation and fibrinolytic systems on inflammation.

CONTINUING EDUCATION QUESTIONS

THROMBOTIC MICROANGIOPATHY (TTP AND HUS): ADVANCES IN DIFFERENTIATION AND DIAGNOSIS
1. Both thrombocytopenic thrombotic purpura (TTP) and hemolytic uremic syndrome (HUS) present with microangiopathic hemolytic anemia. Which set of features best represents TTP?
   a. Verotoxin-producing E. coli, abdominal pain, diarrhea, decreased platelet count, and schistocytes on peripheral smear
   b. Abdominal pain, diarrhea, severely decreased platelet count, and schistocytes on peripheral smear
   c. Abdominal pain, diarrhea, severely decreased platelet count, and negative for schistocytes on peripheral smear
   d. Verotoxin-producing E. coli, abdominal pain, diarrhea, normal platelet count, and positive for schistocytes on peripheral smear

2. Ninety-five percent of these cases are found in children and are associated with infection by verotoxin-producing E. coli:
   a. HUS
   b. TTP
   c. MAHA
   d. TMA

3. The enzyme responsible for cleaving ultra-large von Willebrand factor molecules is:
   a. ULVWF enzyme.
   b. ADAMTS-23.
   c. ULVWF-13.
   d. ADAMTS-13.

4. A deficiency of the enzyme responsible for cleaving ultra-large von Willebrand factor molecules results in:
   a. thrombosis, bleeding and ULVWF molecules.
   b. UL endothelial cells, thrombosis and bleeding.
   c. small von Willebrand subunits and thrombosis.
   d. ULVWF molecules reacting with circulating platelets.

5. Clinically, a deficiency of the enzyme responsible for cleaving ultra-large von Willebrand factor molecules results in:
   a. formation of microthrombi leading to cerebral infarction and renal failure.
   b. a tendency to joint bleeding.
   c. ecchymosis and petechiae.
   d. lack of clinical symptoms making diagnosis difficult.
6. A patient suffering from a deficiency of the enzyme responsible for cleaving ultra-large von Willebrand factor molecules would best be treated by:
   a. a single treatment with plasmapheresis.
   b. removal of the spleen and a single treatment with plasmapheresis.
   c. daily plasmapheresis until the platelet count returns to normal.
   d. infusion of fresh frozen plasma without plasmapheresis.

IS THERE A GENETIC RELATIONSHIP BETWEEN ARTERIAL AND VENOUS THROMBOSIS?
7. Venous thrombosis is generally associated with:
   a. acute myocardial infarction.
   b. pulmonary embolism.
   c. non-hemorrhagic cerebrovascular accidents.
   d. atherosclerotic vascular disease.

8. Arterial thrombosis is traditionally characterized by all of the following EXCEPT:
   a. formation of white clots, rich in platelets.
   b. atherosclerotic vascular injury.
   c. changes in blood composition (thrombophilia).
   d. treatment generally with anti-platelet agents.

9. Which of the following supports the concept that arterial thrombosis and venous thrombosis are linked pathophysiologic processes?
   a. Individuals experiencing a secondary venous thromboembolic events (VTE) have a greater risk of carotid artery plaque formation and atherosclerosis than those experiencing an idiopathic VTE.
   b. Individuals experiencing an idiopathic venous thromboembolic event are more likely to suffer a significant arterial event than the general population.
   c. Individuals experiencing an idiopathic VTE and individuals experiencing a secondary VTE have the same likelihood of having metabolic syndrome.
   d. The Genetic Susceptibility to Thrombosis (GAIT) study showed a stronger correlation with venous thrombosis than arterial trombosis.

CROSS TALK BETWEEN THE INFLAMMATION AND COAGULATION SYSTEMS
10. Which of the following cells is NOT considered a significant contributor to inflammation?
    a. Lymphocytes
    b. PMNs (neutrophils)
    c. Basophils/mast cells
    d. Platelets

11. Which of the following best describes the function of the inflammatory response?
    a. To activate natural killer (NK) cells as part of the host defense
    b. To neutralize or eliminate pathogens (or to destroy injured or necrotic tissue) as part of the host defense
    c. To activate cytotoxic T lymphocytes as part of the host defense
    d. To stimulate antibody production by B lymphocytes

12. All of the following are considered important inflammatory cytokines EXCEPT:
    a. Tumor Necrosis Factor (TNF).
    b. Interleukin-6 (IL-6).
    c. Interleukin-1 (IL-1).
    d. Interleukin-3 (IL-3).

13. The effect of inflammation on the protein C anticoagulant system is to:
    a. decrease thrombomodulin.
    b. decrease C4b binding protein (C4bBP).
    c. increase endothelial cell protein C receptor (ECPR).
    d. increase protein C and protein S.

14. Which of the following statements concerning the effect of inflammation on the hemostatic system is correct?
    a. Induces thrombocytopenia and dysfunctional platelets activation
    b. Increases activity of coagulation inhibitors such as antithrombin (AT) and tissue factor pathway inhibitor (TFPI)
    c. Increases procoagulant activity of the endothelium
    d. Increases activity of the fibrinolytic system
15. Thrombin and other coagulation proteases activate cells via binding to PAR receptors. PAR stands for:
   a. prothrombin associated receptor.
   b. pathogen activated receptor.
   c. plasminogen associated receptor.
   d. protease activated receptor.

16. The major effect of the procoagulant inhibitors, anti-thrombin (AT), activated protein C (APC), and tissue factor pathway inhibitor (TFPI) on inflammation is to:
   a. decrease the production of inflammatory mediators and leukocyte activation.
   b. inhibit the vascular changes seen during the inflammatory response.
   c. stimulate endothelial cells to upregulate thrombomodulin (TM) and endothelial cell protein C receptor (EPCR).
   d. promote leukocyte adhesion and migration.

17. The role of thrombin activated fibrinolysis inhibitor (TAFI) in inflammation is to:
   a. activate proinflammatory mediators such as bradykinin.
   b. inactivate C3a and C5a.
   c. inhibit leukocyte adhesion to the endothelium.
   d. induce leukocyte chemotaxis into the tissues.
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7. a b c d 16. a b c d
8. a b c d 17. a b c d
9. a b c d
FOCUS: PROTEOMICS

Introduction to Proteomics

DELFINA C DOMINGUEZ, ROSANA LOPES, M LORRAINE TORRES

Technological advances in the field of genomics have given rise to the development of a new area called proteomics. Proteomics involves the analysis of all proteins expressed in a genome and uses a combination of sophisticated technologies such as two-dimensional electrophoresis, mass spectrometry and bioinformatics to identify and characterize proteins. This new area offers the potential to discover new biomarkers, improve diagnosis, and improve the prognosis of disease processes. This article presents an overview of proteomics importance and related technologies.

ABREVIATIONS: 2-DE = two-dimensional electrophoresis; IPG = immobilized pH gradient; MALDI-TOF = matrix assisted laser desorption ionization time-of-flight; mRNA = messenger ribonucleic acid; pI = isoelectric point.

INDEX TERMS: mass spectrometry, protein expression, proteome, proteomics, two-dimensional electrophoresis.


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The sequencing of the human genome, which unveiled the total genetic content in eukaryotes and advanced technology, has lead to the development of a new field called proteomics. Proteomics encompasses the analysis of all proteins expressed in a cell. Two dimensional electrophoresis (2-DE) and mass spectrometry (MS) are the main techniques used in proteomics. Documenting protein expression of an organism by 2-DE analysis is not a new concept. In 1970, Kalschmidt and Wittmann separated ribosomal proteins from Escherichia coli by two-dimensional electrophoresis. A few years later, Klose, O’Farrel, and Scheele described 2-DE as the main technique used for the separation of complex protein mixtures into distinct protein spots. The direct study of protein expression began with Anderson and Anderson in 1977 who for the first time analyzed human plasma proteins and anticipated the possibility of identifying all proteins present in the human body. For the past 20 years, 2-DE has been considered the standard technique for analyzing the proteins expressed by cells, tissues and fluids.

Before the genomics revolution, proteins were sequenced by stepwise chemical degradation from the N terminus to the C terminus followed by UV spectroscopy. These procedures were time consuming and only allowed the identification of few proteins. The improvement in efficiency of ionization of molecules by embedding them in a matrix or by electrospray has made possible the rapid identification of thousands of proteins by mass spectrometry. The main goals of proteomics are to identify and characterize altered protein expression, investigate co- and post-translational protein modifications, and correlate protein structure and function with biological activity.
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WHAT IS PROTEOMICS?
Proteomics is the scientific analysis of all the proteins expressed by a genome.\(^1\) A newly developing subdivision of proteomics is “Clinical Proteomics”, which aims to identify proteins involved in pathological processes, to understand how changes in protein expression cause illness, and to develop biomarkers for diagnosis and therapeutic interventions.\(^{10-12}\) Proteins are implicated in almost all biological functions and for this reason are considered to reflect the true status of a cell. The term proteome was employed for the first time by Wasinger and others (1995) and it refers to the total protein complement of a genome.\(^1\)

There are about 25,000-30,000 genes in the human genome and more than 500,000 predicted proteins in the human proteome.\(^{14,15}\) These numbers reflect the high degree of complexity of the human genome and clearly show that many genes encode more than one protein. One of the mechanisms that contribute to such complexity is alternative splicing. This process occurs in eukaryotes. Splicing of a transcribed pre-mRNA from one gene can lead to different mature mRNA molecules, and therefore to different proteins. Genes contain both coding DNA (exons) and noncoding DNA (introns). In certain genes, the variable combination of exons causes a gene to produce different proteins.\(^{16,17}\) An illustration of the alternative splicing process is shown in Figure 1.

Eukaryotic organisms have the ability to regulate gene expression by several mechanisms, and at both the transcriptional and post-translational levels. Mechanisms such as RNA splicing, RNA processing, polyadenylation, and the action of regulatory proteins occur at the transcriptional level. These mechanisms control how, where, and when genes are expressed.\(^{17,18}\) Chemical modifications of proteins like phosphorylation, acetylation, and glycosylation occur at the post-translational level (Figure 2). Post-translational modifications also contribute to the great diversity of proteins and determine cellular localization of proteins, physiological activity, protein interactions, and protein turnover.\(^{19}\) Therefore, measurements of mRNA expression indirectly correlate with protein function, while protein measurement is related directly to function.\(^{20}\) These cellular events explain the importance of proteomics. Despite the great degree of complexity that proteomics presents, it offers more specificity than gene analysis alone.

The study of proteomics has been divided into two areas: 1) expression proteomics and 2) functional proteomics. Expression proteomics encompasses the investigation and evaluation of proteins encoded by active genes in a cell, organ, or tissue.\(^{14}\) Expression proteomics allows the comparison and analysis of differential protein expression profiles between control cells and cells under certain environmental conditions. Therefore, expression proteomics offers the opportunity to compare proteins patterns between health and disease states. On the other hand, functional proteomics analyzes and evaluates protein activity and protein-protein interactions.\(^{14}\) Func-

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Figure 1. Alternative splicing

A gene can contain several exons (dark boxes) and introns (light boxes). Exons can be spliced in different ways. For example, if a gene contains 10 exons, one version of the mRNA transcribed from that gene might contain exons 1-9. Another version of the mRNA might contain exons 1-8, and exon 10. Different mature mRNA can lead to different proteins.\(^{31,32}\)

Figure 2. Eukaryotic gene expression control points

1) Transcriptional control, 2) RNA processing, 3) RNA transport, 4) translational control, 5) mRNA degradation, 6) Protein activity control\(^{32}\)
tional proteomics aims to characterize the information flow of intracellular and extracellular protein networks that link tissues and organs. Functional proteomics can be applied to understand signaling mechanisms involving pathological conditions such as cancer, myocardial disease, and brain damage. Advances in technology made possible the study of proteomics. Sophisticated instrumentation such as mass spectrometry, improvements in liquid chromatography, and 2-DE apparatus have been essential tools in the identification of proteins.

OVERVIEW OF PROTEOMICS TOOLS
Proteomics uses a combination of several techniques including 2-DE, image analyses, mass spectrometry, amino acid sequencing, and bio-informatics to comprehensively resolve, quantify, and characterize proteins. Two-dimensional electrophoresis is a method used to separate proteins by their isoelectric point in the first dimension, and subsequently by molecular weight in the second dimension. Proteins resolved in a 2-DE gel can be stained with Coomassie brilliant blue, silver or fluorescent dyes. Proteins spots purified by 2-DE can be excised from the gel and cleaved through chemical or enzymatic methods. Mass spectrometry is the method of excellence for protein identification. The mass differences in the achieved spectrum among the repeated ions are related to the amino acid composition, which can be used to deduce the peptide sequence. The basic steps in a proteomic analysis are illustrated in Figure 3.

2-DE is a versatile technique that can separate hundreds to thousands of proteins at one time (http://ca.expasy.org/ch2d/protocols/). The protein concentration applied to a gel can vary between several micrograms to one milligram. For low abundance proteins to be detected against a background of abundant proteins, such as albumin in a serum sample, a high-protein-capacity system is required. Capacity is dependent on the volume of the gel. Thinner gels provide better sensitivity for the majority of detection methods, and larger and thicker gels offer increased capacity. The 2-DE technique is considered an essential method for visualization of proteins according to their molecular weight and isoelectric point. The protein sample should be actively or passively rehydrated before first dimension separation. The first dimension utilizes immobilized pH gradient (IPG) that can be applied to different ranges from broad (pH 3-10) to narrow gradients (pH 3-6, 5-8, and 7-10). Proteins migrate in isoelectric focusing gels to their neutral isoelectric point (pI), where the protein has zero net charge. First-dimensional gels are loaded onto the second-dimensional polyacrylamide gel slabs after equilibration. For the second dimension, polyacrylamide gels are used in different percentages according to molecular weight of proteins to be separated. Several stains can be used to visualize proteins after 2-DE. Stains more commonly used are Coomassie brilliant blue, (detection limit = 8 ng-16ng) and silver staining (detection limit = 2 ng-4ng). In addition, fluorescent dyes can be utilized such as: Sypro Red, Sypro Orange, Sypro Tangerine

Figure 3. Two-dimensional electrophoresis technique

Protein samples are separated according to their isoelectric point (1st dimension) followed by their molecular weight (2nd dimension). Protein identification is commonly performed by mass spectrometry.
Mass spectrometry (MS) is a powerful technique that identifies unknown compounds and quantifies known compounds and reveals the chemical structure of molecules (http://www.asms.org/whatisms/p1.html). Mass spectrometry measures the mass to charge ratio of ionized molecules and proteins are identified based on their spectrum features. There are different MS techniques used for the identification of proteins. The most commonly used are MALDI-TOF (matrix-assisted laser desorption ionization-time-of-flight), ESI-MS/MS (electrospray ionization tandem mass spectrometry), LC-MS/MS (liquid chromatography tandem mass spectrometry), SELDI (surface enhanced laser desorption ionization), and ICAT (isotope coded affinity tags). The principle and applications of these techniques will be discussed in the proteomics technologies section.

CONCLUSION

Advances in genomics (genome sequences data bases, microarray technology) and technology improvements (mass spectrometry instrumentation) have given rise to a new area of study called proteomics. This field offers the potential to examine and understand protein expression in health and disease, to improve diagnosis, and the possibility to provide a “personalized” therapeutics. This article presents a brief introduction to the field of proteomics presenting history, background information and a review of the technology employed.

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Clinical Practice
Wayne Gade and Brooke Robinson, for their articles A Brief Survey of Aquaporins and Their Implications for Renal Physiology and CLS Meets the Aquaporin Family: Clinical Cases Involving Aquaporin Systems, published in the Spring 2006 issue of Clinical Laboratory Science.

Research and Reports
Rebecca Laudicina, for her article Searching for Hereditary Hemochromatosis, published in the Summer 2006 issue of Clinical Laboratory Science.

Focus
FOCUS: PROTEOMICS

Proteomics Technology

DELFINA C DOMINGUEZ, ROSANA LOPES, M LORRAINE TORRES

Proteomics techniques are essential tools for protein detection and characterization. Besides several advances in the proteomics field, the two-dimensional electrophoresis (2-DE) technique is the most important method for protein separation. The combination of 2-DE technique, new advances in mass spectrometry and bioinformatics promises to unveil protein function and pathological mechanisms of disease.

ABREVIATIONS: 2-DE = two-dimensional electrophoresis; CHAPS = (3-[ (3-Cholamidopropyl)-Dimethylammonio]-1-Propane Sulfonate); ESI - MS = electrospray ionization mass spectrometry; ICAT = isotope-code affinity tag; IPG = immobilized pH gradient; LC-MS = liquid chromatography mass spectrometry; MALDI = matrix assisted laser desorption ionization; MALDI-TOF = matrix assisted laser desorption ionization time-of-flight; mRNA = messenger ribonucleic acid; pI = isoelectric point; SELDI-MS = surface-enhanced laser desorption/ionization time-of-flight.

INDEX TERMS: ICAT; LC-MS, MALDI; protein expression; proteomics; SELDI-MS; two-dimensional electrophoresis.


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Delfina C Domínguez PhD is the Focus: Proteomics guest editor.

LEARNING OBJECTIVES
1. Recognize the different tools applied to proteomics analysis.
2. Differentiate the procedures utilized in proteomics studies starting from protein sample preparation to protein identification methods.
3. Identify the steps needed for analysis of protein expression by software packages.
4. Distinguish the methods employed for qualitative and quantitative protein identification.

After geneticists sequenced the human genome and learned of the poor correlation between mRNA and protein numbers, special attention was given to the gene products, the proteins. From approximately 25,000 predicted genes in human beings, it is expected that there will be more than 500,000 protein products as a result of splicing and protein modifications. Proteomics analyses utilize several techniques for protein separation, detection, and identification. For separation of simple and complex protein mixtures, two-dimensional electrophoresis (2-DE) is the technique of choice. Several staining procedures can be applied to 2-DE gels for protein detection. Mass spectrometry is considered the best method for protein identification.

TWO-DIMENSIONAL ELECTROPHORESIS (2-DE)

Sample preparation

A wide variety of samples can be utilized to identify and characterize proteins in proteomic studies. Some of these samples include: samples fractionated from an organism (eukaryote or prokaryote), tissue, cell lysates, and physiological fluids. A careful research design should be planned to acquire a meaningful representation of the proteins of interest. It is essential to ensure that proteins are soluble in order to...
obtain an accurate protein analysis. Protein solubilization is achieved utilizing chaotropes (urea), reducing agents (dithiothreitol), detergents (CHAPS, Triton-X), buffers (Tris), and <0.2% ampholytes. In addition, size, charge, and isoelectric point of proteins of interest are important characteristics for sample preparation. Moreover, to reduce complexity of protein data, subcellular fractionation methods can be used to analyze proteins from specific organelles such as endoplasmic reticulum, mitochondria, nucleus or cell wall and membrane fractions from microorganisms. Some additional methods such as selective fractionation and chromatography can also be utilized to obtain a specific set of proteins from a complex mixture. A good sample preparation prevents protein aggregation and enzymatic and chemical degradation. It also eliminates or digests nucleic acids producing a standardized protein sample. The Bradford and Lowry methods are commonly used for protein concentration measurements.

2-DE - first dimension
The first step toward protein separation is to determine its isoelectric point. The isoelectric focusing of proteins can be determined using immobilized pH gradients or ampholytes. In general, the pH gradient used for protein separation is in the range of pH 3-12. To obtain a reference map displaying the majority of proteins, a broad-range of pH gradient (pH 3-10) should be employed, but if a special category of proteins with a specific pH range is of interest, narrow pH gradients will increase the gel resolution and improve the detection of low abundance proteins. Nonlinear pH gradients are used to improve visualization of proteins in the middle of the pH range. The estimation of the isoelectric point of a protein spot can be obtained through the position of a known protein standard present in the same pH gradient. Rehydration of immobilized pH gradient (IPG) requires a minimum of 11 hours. During active rehydration a low voltage is applied while the protein sample is absorbed by the IPG. The electric current helps the protein absorption into the IPG but there is possibility of loss of low abundance proteins during this process. In contrast, during passive rehydration the protein sample is absorbed into IPG without presence of an electric field. Sample application by cup loading should be avoided due to the tendency of artifacts production. The voltage conditions and resolution of proteins for first dimension are dependent on the pH gradient, length of IPG strip, and electrical field applied (Figure 1).

2-DE – second dimension
In the second dimension, all proteins separated in the first dimension will be resolved according to their molecular weight. Different percentages of acrylamide determine the pore size of gels. The size of the pores created in the gel is inversely related to the amount of acrylamide used. For example, an 8% polyacrylamide gel will have larger pores in the gel than a 15% polyacrylamide gel. Gels with a low percentage of acrylamide are typically used to resolve large proteins and high percentage gels are used to resolve small proteins.

Gradient gels have different concentrations of acrylamide allowing better resolution of proteins. Gradient gels are cast with a higher concentration of acrylamide at the bottom of the gel and a low concentration of acrylamide at the top of the gel. The applications of gradient gels include the determination of protein molecular weight and the separation of molecules which co-migrate on uniform gels.

Single percentage acrylamide gels will favor the separation of proteins in a narrow pH range. The run time for second dimension depends on size of gel and may range from 30 minutes for mini-gels to six hours for larger gels.

Protein detection in gel
The staining choice for 2-DE gels depends on the protein sample characteristics. For example, if low-abundance proteins are targeted, 0.1-1mg/mL of protein sample should be loaded, and a very sensitive staining should be employed.
such as silver or fluorescent dyes. Double staining can be performed with Coomassie brilliant blue and silver or Coomassie brilliant blue and fluorescent dyes. This procedure allows investigation of protein staining sensitivity throughout the gel once proteins interact differently with diverse types of staining.

**IMAGING AND PROTEIN ANALYSIS METHODS**

A variety of equipment can be utilized for acquisition of 2-DE gel imaging such as cameras, scanners, and densitometers. Different software packages are available for analysis of protein expression such as PDquest (Bio-Rad), ImageMaster 2D (GE Healthcare), Progenesis Workstation (Nonlinear Dynamics), and Melanie (Geneva Bioinformatics).

A classical 2-DE analysis is based on five features: 1) Normalization, 2) protein spot detection, 3) generation of a matched set of images, 4) relative quantitation of protein spots (Figure 2), and 5) statistical analysis of data. Several web sites are available for matching peptides to known protein sequences such as MASCOT (http://www.matrixscience.com/), PepMAPPER (http://www.molecularstation.com/bioinformatics/link/detail/link-453.html), Aldente Peptide Mass Fingerprinting (http://ca.expasy.org/tools/aldente/), and ProteinProspector (http://prospector.ucsf.edu/).

**PROTEIN IDENTIFICATION PROCEDURES**

**Qualitative mass spectrometry analysis**

Advances in instrumentation have made mass spectrometers an essential tool for protein identification in proteomic analyses. Often, after 2-DE the protein spot is excised and subjected to in-gel trypsin digestion. The fragmented peptides are identified by mass spectrometry. Trypsin cleaves proteins on the C-terminal side of lysine and arginine residues. The mass spectra data of peptides identified are matched to known protein database sequences.

In mass spectrometry (MS) the protein molecules are ionized and subsequently separated according to their mass-to-charge ratio. The separated ions are then detected by the analyzer. For many years MS was used only for small and heat stable compounds due to the difficulty in ionizing the molecules effectively and the inability to transfer the ions from the condensed phase into the gas phase without excessive fragmentation.

Two techniques that revolutionized MS were developed during the 1980s. These techniques were matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). MALDI is known as “soft ionization” and utilizes a matrix, alpha-cyano-4-hydroxycinnamic acid and 2, 5 dihydroxybenzoic acid where peptides are embedded. Proteins to be analyzed are vaporized by a pulse-UV laser beam. Ionized proteins are accelerated in an electrical field and their mass-to-charge ratio measured. This is the key feature of this ionization method. This method is widely used to identify tryptic peptides from simple protein mixtures because sample identification can be done in less than two hours depending on the number of samples, it is sensitive to the femtomole range and inexpensive. The most common MALDI MS is MALDI-TOF (time-of-flight) (Figure 3). The mass-to-charge ratio in TOF analyzers is deduced from the flight time the ionized protein requires to travel through a tube of specific length under vacuum. TOF analyzers give high mass accuracy and resolution. Another variety of MALDI is the MALDI-QqTOF.

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**Figure 2. Relative quantitation of mitochondrial proteins map**

Map shows position and standard spot numbers (SSP) of quantitation analysis from PDQuest (version 7.1) software. Position of proteins spots down-regulated by 2.0 fold or greater are shown in black. Mr = molecular weight; pI = isoelectric point.
In this special case a MALDI-TOF spectrometer is connected to a quadrupole, which guides the ions to the TOF analyzer where the mass analysis takes place. If the identity of a protein cannot be obtained by MALDI-TOF from known protein databases, the electrospray ionization-MS/MS (ESI-tandem MS) method should be employed. In ESI-MS/MS, the protein sample is in liquid phase. The sample is pumped through a hypodermic needle at high voltage. The ionized molecules are then analyzed by the mass spectrometer (Figure 4). ESI-MS/MS offers the advantage of providing the peptide molecular weight plus sequence information. Therefore, it is much easier to identify the protein. Still, ESI-MS/MS has some disadvantages compared to MALDI. Sample conditioning plays an important role. ESI-MS/MS analysis time is limited since the liquid sample is consumed during the measurements. Also ESI-MS/MS is concentration-sensitive and requires desalted samples. Further, one to five samples may cost $450.00. The presence of detergents, metals, and salts may cause problems and signal suppression. These problems can be overcome by combining liquid chromatography (LC) with MS analysis. During LC-MS the protein sample is eluted from a chromatographic column and then analyzed by mass spectrometry. This type of analysis is especially useful when complex mixtures are analyzed.

Quantitative mass spectrometry analysis
To quantify a protein sample, stable isotopes are employed to label digested protein. The isotope-coded affinity tagging (ICAT) developed by Gygi and others (1999) is able to simultaneously analyze and accurately identify and quantitate proteins from complex mixtures. It is possible to compare the proteins present in two proteomes. For example, the protein samples are mixed with the ICAT reagents, which consist of a cysteine reactive moiety, a biotin affinity tag for isolation and purification, and a linker region consisting of hydrogens (light tag) or deuteriums (heavy tag). The protein sample then is subjected to tryptic digestion followed by LC-MS/MS. Proteins are quantified based on signal intensities of identical peptides labeled with either the heavy or the light tag form the ICAT reagent. This method has the advantage of omitting the 2-DE separation step. However, the main limitation of the ICAT method is that it requires the labeling of a free cysteine residue on the protein. Therefore, proteins lacking a free cysteine residue will not be identified. This method is a low throughput method in which only two samples can be done per run.

Another quantitative MS method is the surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry, which has a quantitative protein chip and it is known as SELDI-TOF-MS-based Protein Chip®. The principle of this method is very similar to the MALDI. In both methods the peptides are cocrystalized on a matrix or surface and peptides are vaporized by pulsed-UV laser beam. The ionized proteins then are accelerated in an electrical field and the mass-to-charge ratio is deduced from their velocity. In SELDI protein solutions are applied to a protein chip array that has a specific chromatographic surface. The proteins interact with the array surface and are selectively bound according to their surface interaction. Once the proteins are bound, the surface is washed with a buffer solution, ensuring sample cleaning. Subsequently a laser beam causes desorption and ionization of proteins. A specific laser beam scans the entire protein chip and performs repeated readings of a single spot. The laser never reads the same position twice. An average spectrum is obtained and good correlation between the signal intensity and concentration of the analyte is obtained for the different protein samples. The SELDI-TOF ProteinChip® system is limited to bound proteins and is unsuitable for analysis of proteins >100 kDa molecular weight.

CONCLUSIONS
2-DE and MS analyses are crucial technologies in the study of proteomics. Great advances in instrumentation developed in the last few years have made it possible to rapidly and accurately analyze complex protein mixtures. A wide variety of systems have been created including quantitative MS.
Although the accelerated advancement in MS technology has been driven by protein research, it can be anticipated that MS will also be used for the analysis of other molecules such as lipids and carbohydrates. Specific challenges still remain in the areas of protein quantitation and software development for data acquisition, data interpretation, and presentation. Further improvement in MS technology will make MS an even more powerful tool for proteomic analysis.

Clin Lab Sci encourages readers to respond with thoughts, questions, or comments regarding this article. Email responses to ic.ink@mchsi.com. In the subject line, please type “CLIN LAB SCI 20(4) FOCUS: PROTEOMICS”. Selected responses will appear in the Dialogue and Discussion section in a future issue. Responses may be edited for length and clarity. We look forward to hearing from you.

REFERENCES
FOCUS: PROTEOMICS

Proteomics: Clinical Applications

DELFINA C DOMINGUEZ, ROSANA LOPES, M LORRAINE TORRES

The word proteomics was coined in 1997 to describe the changes in all proteins expressed by a genome. Several sophisticated techniques including two-dimensional electrophoresis, imaging, mass spectrometry, and bioinformatics are used in proteomics to identify, quantify, and characterize proteins. Clinical proteomics is the application of proteomics techniques to the medical field. The main aim of this methodology is to identify proteins involved in pathological processes and to understand how illness can lead to altered protein expression. Clinical proteomics offers the opportunity and the potential to develop new diagnostic and prognostic tests, to identify new therapeutic targets, and eventually to allow the design of individualized patient treatment. Here we present an overview of proteomics applications to the study of disease and its potential to improve diagnosis and prognosis.

ABREVIATIONS: 2-DE = two-dimensional electrophoresis; DCM = dilated cardiomyopathy; ELISA = enzyme-linked immuno-absorbent assay; HCC = hepatocellular carcinoma; IL-6 = interleukin-6; LC/MS/MS = liquid chromatography mass spectrometry; MS = mass spectrometry; PSA = prostate-specific antigen; SELDI-MS = surface-enhanced laser desorption/ionization time-of-flight.

INDEX TERMS: cancer; clinical proteomics; protein expression; two-dimensional electrophoresis.


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LEARNING OBJECTIVES
1. Present an overview of proteomics methodology.
2. Comprehend how proteomic methodology is applied in the medical field.
3. Describe the potential application of clinical proteomics in the development of new biomarkers in diagnostic and prognostic tests.

Clinical proteomics aims to identify proteins involved in pathological processes and to evaluate changes in protein expression during illness. Moreover, clinical proteomics offers technical capabilities to develop biomarkers for diagnosis and therapeutic interventions. Proteomics analysis utilizes multiple methodologies to characterize and identify altered proteins as a result of disease. One of the most common techniques is two-dimensional electrophoresis (2-DE), which is used to compare the protein expression among healthy individuals and diseased subjects. In addition, a combination of immunochemical analyses of proteins, bioinformatics (the use of computational techniques to extract meaning from biological data), and different mass spectrometry techniques are used for protein detection and identification.

The diagnosis of various disease states such as cancers and cardiomyopathies is currently based on the detection of single proteins such as troponin, prostate specific antigen (PSA), CA-125, and others. Proteomic analysis examines thousands of proteins at one time allowing the detection of specific protein patterns expressed as a consequence of abnormal cellular function or cellular interactions. Therefore, poten-
Potential biomarkers developed as a result of proteomics analysis will have higher sensitivity and specificity since multiplexed panels of clinical tests will measure the altered proteins.

As often happens when new technology is introduced there are many expectations and hopes. There is no exception for clinical proteomics, however, major challenges still remain. One major challenge is to optimize the detection of low abundance proteins (cytokines, transcription factors, and cell-signaling proteins) in tissue, plasma, and body fluids, which are found in the nanogram and picogram range. Moreover, further improvement in software development for data acquisition and interpretation is needed. A good understanding of data management, correlation, interpretation, and validation is crucial to obtain accurate and meaningful results.

Here we present some examples of how clinical proteomics is being used to study disease, and its potential applications in biomarker discovery.

**CANCER DIAGNOSTICS**

No standardized screening test is available to reliably detect ovarian cancer. Most women with ovarian cancer are diagnosed at the latest stages of the disease with a five year survival rate of 35%. However, if this cancer could be detected earlier the chances of survival would increase dramatically to >90%. The tumor marker CA-125 is often used to screen for ovarian cancer. However, this test lacks sensitivity and specificity as Ca-125 may be elevated in other physiologic and pathologic conditions. A recent proteomics study has shown promising results. Serum samples of 50 patients with ovarian cancer and 50 unaffected patients were analyzed by SELDI-TOF mass spectrometry. Results showed different protein patterns in cancer patients compared to unaffected individuals with 100% sensitivity and 95% specificity. However, the protein profile observed in the cancer group was not identified. In addition, the population tested was small and there were reproducibility problems. Identification of aberrant proteins expressed as a result of cancer is of primary importance for the development of immunoassays and future markers.

Another cancer that has been screened for biomarkers using a proteomic approach is hepatocellular carcinoma (HCC). Protein expression of patients with HCC has been analyzed using serum auto-antibodies that showed cross-reactivity with proteins from the patient’s tumor. Three proteins were found to be overexpressed in this analysis: hsp 70, peroxidoxin, and manganese-superoxide dismutase (Mn-SOD). These proteins have been considered potential markers for HCC.

Lung cancer causes more deaths than the combination of the three most common cancers: colon, breast, and prostate. Patients affected by this disease have a five year survival rate when the disease is still localized. However, only 24% of lung cancer cases are diagnosed at an early stage. Proteomics analysis has shown that Napsin A protein was only expressed in patients with primary lung adenocarcinoma. Therefore, this protein has been used as a potential biomarker to differentiate the primary form of lung adenocarcinoma from its metastatic form.

**CARDIOVASCULAR DISORDERS**

Among several heart diseases, cardiomyopathies have received special attention from proteomics researchers. In 1998, a novel finding by Corbett and others was the decreased expression of 88 myocardial proteins in humans with dilated cardiomyopathy (DCM). These results were confirmed later by other investigators. Some of these low abundant proteins might be considered as useful diagnostic and/or prognostic markers for DCM.

Proteomics can be a key tool for the prognosis of cardiac allograft rejection. Tissue rejection is one of the major problems after cardiac transplantation. Post-transplant endomyocardial biopsies have shown that 100 proteins were overexpressed after cardiac transplantation; however, only thirteen proteins had cardiac-tissue specificity. Of those, two proteins (alpha beta-crystallin and tropomyosin) could be measured in patient’s serum presenting cardiac rejection after three months. This is an example of how powerful proteomics techniques can be and their applications in biomarker discovery.

**MICROBIOLOGY**

In order to simplify proteomics methodology microbiologists have used bacterial protein-enriched fractions instead of the whole bacterial proteome. This approach examines a specific component of the bacterial cell. For example, examining the cell membrane and/or extracellular proteins of an organism is of great interest since these proteins are often involved in host-pathogen interactions. Blonder and others used liquid chromatography coupled with mass spectrometry to analyze the *Pseudomonas aeruginosa* membrane subproteome (selected fraction of proteins from an organism). This study identified 786 proteins. Some of these proteins were integral...
and outer membrane proteins involved in adaptation and antibiotic resistance. Although the results of this study are subject to further confirmation, many proteins identified may be targets for development of novel drug therapies.

In an effort to improve immunodiagnostics and vaccines for Mycobacterium tuberculosis, Bahk and others examined M. tuberculosis proteins secreted in culture by 2-DE. Eight proteins were identified by liquid chromatography and tandem mass spectrometry (LC/MS/MS). All proteins were cloned and over-expressed in E. coli cells. Three proteins (rRV3369, rRv3874, and rRv0566c) were selected for pre-screening and potential serodiagnostic antigens. Sera from 100 tuberculosis patients and 100 sera from healthy controls were analyzed by ELISA. Results from the analysis showed that the antigens rRV3369 and rRv3874 had 60% and 74% sensitivity and 96% and 97% specificity, respectively. These proteins have the potential to be used in the serodiagnosis of tuberculosis.

Proteomic analysis has been used to study protein expression in virulent and avirulent strains, interaction of bacteria with eukaryotic cells grown in vitro, the host immune response to infection, and drug resistance of microorganisms.

Based on the information presented, it is clear that the application of proteomics to the medical field has a great potential for the improvement of diagnostics and therapeutics. Nevertheless, there are challenges that need to be overcome. Blood is perhaps one of the most complex proteomes because in addition to proteins normally found in plasma other proteins that are not normally present may be found. These additional proteins may be released from tissue in response to injury or a disease state. Protein concentration in plasma varies dramatically. Among the most abundant proteins in plasma are albumin and the globulins, which are present in the milligram per milliliter range, in contrast to cytokines (IL-6), which are found in picograms per milliliter. This extreme difference poses a problem during proteomic analysis. Therefore, in proteomic studies elimination of highly abundant proteins is required to facilitate analysis, however, during elimination of the abundant proteins, such as albumin, low abundance proteins that bind albumin and that may be of interest for biomarker discovery may also be removed.

It is necessary to have a good understanding of the variability sources that may contribute to error such as pre-analytical, analytical, and biological variation. Pre-analytical variability may be introduced during specimen collection and manipulation, pipetting, and dilution of samples. Careful consideration should be given to specimen collection using different tube type (whole blood/plasma/serum), coagulation times, and storage conditions. Analytical variability may occur in inaccurate calibration of instruments (MS/2-DE), standardization of output, and appropriate controls and proper bioinformatics methodology. In addition, it is important to account the biological variability due to gender, age, race, and fluctuations that may occur daily within an individual (biorhythm, fasting, time of the day). All these variables may induce changes in that are not pathological in nature but that have to be differentiated from a pathologi-cal-induced process.

CONCLUSION
Clinical proteomics offers the promise of biomarker discovery and early detection, diagnosis and prognosis of disease, but major challenges still remain. Further advances in technology are needed to eliminate proteomics deficiencies and augment its contributions to the medical field.

Clin Lab Sci encourages readers to respond with thoughts, questions, or comments regarding this article. Email responses to ic.ink@mchsi.com. In the subject line, please type “CLIN LAB SCI 20(4) FO DOMINGUEZ”. Selected responses will appear in the Dialogue and Discussion section in a future issue. Responses may be edited for length and clarity. We look forward to hearing from you.

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Continuing Education Questions

FALL 2007

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LEARNING OBJECTIVES
Upon completion of this section, the reader will be able to:
1. compare and contrast gene expression versus protein expression.
2. discuss the purpose and goals of proteomics.
3. identify the steps followed in a proteomic analysis.
4. describe the main techniques used in proteomics.
5. recognize the different tools applied to proteomics analysis.
6. differentiate the procedures utilized in proteomics studies starting from protein sample preparation to protein identification methods.
7. identify the steps needed for analysis of protein expression by software packages.
8. distinguish the methods employed for qualitative and quantitative protein identification.
9. present an overview of proteomics methodology.
10. comprehend how proteomic methodology is applied in the medical field.
11. describe the potential application of clinical proteomics in the development of new biomarkers in diagnostic and prognostic tests.

CONTINUING EDUCATION QUESTIONS

INTRODUCTION TO PROTEOMICS
1. The scientific approach to analyze all proteins expressed by a genome is known as:
   a. the Human Genome Project.
   b. proteome.
   c. proteomics.
   d. gene analysis.

2. The main goal of proteomics is to:
   a. identify and characterize altered protein expression.
   b. investigate co-and posttranslational protein modification.
   c. correlate protein structure and function with biological activity.
   d. all of the above.

3. Objectives of clinical proteomics are all of the following EXCEPT:
   a. identify proteins involved in pathological processes.
   b. identify processes of protein expression.
   c. understand how changes in protein expression cause illness.
   d. develop biomarkers for diagnosis and therapeutic interventions.

4. In certain genes, the combination of exons can make a gene become active and each combination may result in a different protein. This process is known as:
   a. alternative splicing.
   b. RNA splicing.
   c. RNA processing.
   d. gene processing.

5. Eukaryotic gene expression and regulation at the transcriptional level occur by all of the following EXCEPT:
   a. RNA splicing.
   b. RNA processing.
   c. polyadenylation.
   d. chemical alteration.

6. Post-transcriptional modifications of proteins occur by all of the following EXCEPT:
   a. acetylation.
   b. glycosylation.
   c. polyadenylation.
   d. phosphorylation.

7. Expression proteomics differs from functional proteomics in that:
   a. expression proteomics analyzes and evaluates protein activity.
   b. expression proteomics analyzes protein-protein interactions.
   c. expression proteomics compares the expression profiles of protein patterns.
   d. expression proteomics understands signaling mechanisms involving pathological conditions.
8. Which of the following techniques has been considered for the past 20 years the standard technique for analyzing proteins?
   a. UV spectroscopy
   b. Two-dimensional electrophoresis
   c. Stepwise chemical degradation
   d. None of the above

9. The chronological order for identification of proteins using 2-DE is:
   a. protein separation by mass spectrometry followed by separation by isoelectric point followed by molecular weight.
   b. protein separation by molecular weight followed by separation by isoelectric point followed by mass spectrometry.
   c. protein separation by mass spectrometry followed by separation by molecular weight followed by isoelectric point.
   d. protein separation by isoelectric point followed by separation by molecular weight followed by mass spectrometry.

10. The mass spectrometry techniques used for the identification of proteins are all the following EXCEPT:
    b. Liquid Crystal Multiple Sequence Mass Spectrometry (LC-MS-MS).
    c. Electrospray Ionization tandem Mass Spectrometry (ES-MS/MS).
    d. Surface Enhanced Laser Description Ionization (SELDI).

PROTEOMICS TECHNOLOGY
11. The most important factor during protein sample preparation is:
    a. nucleic acid digestion.
    b. protein solubilization.
    c. protein aggregation.
    d. enzymatic degradation.

12. The main goal of protein sample preparation is to:
    a. increase protein aggregation and use reducing agents such as chaotropes and detergents to improve solubility.
    b. avoid protein sample pre-fractionation and increase the solubility of the sample.
    c. increase protein solubility, reduce complexity, and eliminate nucleic acid.
    d. increase solubility and complexity while enhancing enzymatic and chemical degradation.

13. Methods used to reduce protein complexity are all of the following EXCEPT:
    a. subcellular fractionation.
    b. selective fractionation.
    c. chromatography.
    d. pre-fractionation.

14. The mass spectrometry techniques used for the identification of proteins include all of the following EXCEPT:
    b. Electromagnetic Ionization – MS/MS (EM-MS).
    c. Isotope-coded Affinity Tagging (ICAT).
    d. Surface Enhanced Laser Description Ionization (SELDI).

15. All of the following are true concerning first dimension 2-DE EXCEPT:
    a. cup loading of the sample should be used to decrease production of artifacts.
    b. the isoelectric point of the protein is determined using immobilized pH gradients.
    c. a broad-range pH gradient is used to obtain a reference map of the majority of proteins.
    d. nonlinear pH gradients improve visualization of proteins in the middle of pH range.

16. The five chronological steps in a classical 2-DE analysis are:
    a. protein spot detection, matchset generation, normalization, protein spot quantitation, and statistical analysis.
    b. normalization, protein spot detection, matchset generation, quantitation of protein spots, and statistical analysis.
    c. matchset generation, protein spot detection, protein spot quantitation, normalization, and statistical analysis.
    d. protein spot detection, normalization, protein spot quantitation, matchset generation, and statistical analysis.

17. The chronological order of the three basic steps involved in mass spectrometry is:
    a. analyzer detection, ionization of protein molecules, and mass-to-charge ratio separation.
b. mass-to-charge ratio separation, analyzer detection, and ionization of protein molecules.
c. ionization of protein molecules, analyzer detection, and mass-to-charge ratio separation.
d. ionization of protein molecules, mass-to-charge ratio separation, and analyzer detection.

18. What is the major difference in protein analysis between MALDI and ESI?
   a. MALDI protein analysis occurs in solid phase and ESI analysis occurs in liquid phase.
   b. MALDI analysis uses a liquid phase and ESI analysis occurs in a gas phase.
   c. MALDI analysis is performed in a liquid phase and ESI occurs in a solid phase.
   d. MALDI analysis is performed in a gas phase and ESI occurs in a liquid phase.

19. All of the following are true concerning ICAT EXCEPT:
   a. that it omits the 2-DE separation.
   b. that it requires free cysteine residues on the protein being labeled.
   c. that it is unable to quantitate proteins from a complex mixture.
   d. that it is possible to compare the proteins present in two proteomes.

20. In second dimension 2-DE all proteins previously separated via first dimension 2-DE are subsequently separated according to their molecular weight. This is accomplished by using:
   a. uniform concentration of acrylamide gels for broad pH range.
   b. different concentrations of acrylamide gels.
   c. short run times for large gels.
   d. long run times for mini-gels.

PROTEOMICS: CLINICAL APPLICATIONS

21. Clinical proteomics offers the opportunity to:
   a. develop new diagnostic tests.
   b. develop new prognostic tests.
   c. identify new therapeutic targets.
   d. all of the above.

22. Which of the following is the most common technique used in proteomics technology?
   a. Immunochemical electrophoresis
   b. Bioinformatics
   c. Mass spectrophotometry
   d. Two-dimensional electrophoresis

23. All of the following are challenges to proteomic clinical application except:
   a. optimization of low abundance protein detection.
   b. analysis of bacterial virulent strains of protein expression.
   c. software development for data acquisition.
   d. correlation, validation, and interpretation of clinical data.

24. Proteomic technology includes which of the following?
   a. Separation of protein by their molecular weight and isoelectric point
   b. Removal and identification of specific proteins
   c. Enzymatic digestion and ionization of protein particles
   d. a, b, and c

25. Many tumor markers lack specificity and sensitivity. In contrast, the use of proteomics applications in diagnosis and prognosis of cancer offers a more reliable biomarker by detecting:
   a. albumins and globulins.
   b. aberrant proteins.
   c. carrier proteins.
   d. proteins with high mass/charge ratio.

26. Alpha-beta crystallin and tropomyosin are examples of clinical proteomic application correlates with:
   a. cardiac allograft rejection.
   b. ovarian cancer.
   c. lung cancer.
   d. cardiomyopathy.

27. Proteomics methodology in microbiology can be simplified by the use of:
   a. bacterial organelles.
   b. bacterial cloning.
   c. bacterial protein enriched fractions.
   d. whole bacteria protein.

28. Important issues in the clinical application include all of the following except:
   a. specimen collection and processing.
   b. standardization of protein output.
   c. patient’s biorhythm.
   d. accurate calibration of instruments.
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Circle correct answer.

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AB = Abstract
CP = Clinical Practice
DD = Dialogue and Discussion
FO = Focus
RR = Research and Reports
WB = Washington Beat

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