Counterpoint The Vision for a New Diagnostic Paradigm

Emanuel Petricoin III[1] and Lance A. Liotta[2*]

Dr. Diamandis raises concerns about the technical feasibility and biological validity of using mass spectroscopy to profile serum proteomic biomarker patterns. Scientific skepticism and debate are essential to the progress of science. However, the pipeline of approved new markers is drying up *(1, 2)*. Currently, serum proteomic pattern analysis has the potential to discover useful biomarkers faster than any existing technology. Ultimately, the deciding factor for any new diagnostic technology is true patient benefit.

Serum mass spectroscopic proteomic pattern diagnostics is a rapidly expanding field of study. Since our initial publication *(3)* showing the feasibility for ovarian cancer detection, other laboratories have confirmed and extended this concept *(4–8)*. The growing excitement for this new approach goes far beyond the adoption of mass spectroscopy as a diagnostic instrument. Indeed, mass spectroscopy is well established as a routine clinical diagnostic tool. It has been successfully used for many years for neonatal metabolic disorder screening, where the sensitivity and reproducibility of this technology are comparable to those of other clinical assay methods *(9, 10)*. The true scientific goal of serum proteomic pattern analysis is improved biomarker discovery.

There is a great need to discover novel biomarkers and translate them to routine clinical use *(1)*. Conventional differential display technologies (gene arrays, two-dimensional polyacrylamide gel electrophoresis, and others), followed by antibody production, validation, and ELISA testing, are inherently costly and laborious with long cycle times between discovery and clinical implementation. The paucity of new Food and Drug Administration-approved or even "homebrew"-based analytes is driving investigators to break out of this cycle. Mass spectroscopic serum proteomic pattern analysis can sort through tens of thousands of potential biomarkers in the time it takes to read this sentence.

The general hypothesis is that patterns of low-molecular-mass biomarkers in the blood specifically reflect the underlying pathologic state of an organ, even at a distance. Moreover, this pattern of features can achieve a higher accuracy and specificity compared with any single biomarker alone. Although single analytes, such as kallikrein 6 *(11)* or osteopontin *(12)*, may show some discriminatory power for cancer detection in small study sets, it is unclear that any single analyte can detect cancer with high specificity across large heterogeneous populations. The low-molecular-weight serum proteome contains an enormous wealth of biomarker information that has not been explored. Moreover, mass spectroscopy exhibits optimal performance in the low-molecular-mass range. Mathematically, a pattern of multiple biomarkers may contain a higher level of discriminatory information compared with a single biomarker alone, particularly across large heterogeneous patient populations, and for complex multistage diseases such as cancer.

Dr. Diamandis raises concerns about the reproducibility of generating patterns, the relatively poor sensitivity of surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry compared with traditional clinical testing methods, and the unknown "epiphenomena" behind the specificity of the biomarker patterns themselves.

Because cancer cells themselves are deranged host cells, we may never find a true cancer-specific molecule. On the other hand, the complex proteomic signature of the tumor-host microenvironment may be unique and may constitute a biomarker amplification cascade. The specificity of this unique microenvironment can be mirrored by a catalog of low-molecular-weight proteins and peptides, including specifically cleaved, or otherwise modified, proteins produced in sufficient abundance to be detected by current mass spectrometry platforms. The subtlety of these changes can be detected by new pattern recognition algorithms that profile the relative signals of an entire constellation of events simultaneously. The underlying "epiphenomena" may in fact be proteolytic events, induced host proteins, or posttranslational modifications distant from the cancer itself but specific for the event nonetheless. This hypothesis continues to be reinforced as inflammatory conditions, benign pathologies, and other disease states are found to be associated with ion signatures not classified as cancer-like. "Epiphenomena" thus are clearly in the eye of the beholder.

Mass spectroscopy as a clinical analytical method has many unique attributes that no ELISA can achieve at this

[1] NCI-FDA Clinical Proteomics Program, Office of the Director, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892.

[2] NCI-FDA Clinical Proteomics Program, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892.

*Author for correspondence.

Received April 18, 2003; accepted May 23, 2003.

time. In addition to the speed of mass spectroscopy, ions can be precisely identified without the need for antibody development or a priori amino acid sequencing. This agnostic approach affords the experimentalist an approach to disease detection without bias about the source or identity of the markers. Mass spectroscopy can differentiate clipped or modified versions of molecules with extremely high speed and resolution. If the biomarker were a cleaved version of a larger, abundant protein, it may be nearly impossible to generate antibodies that recognize the cleaved version and do not cross-react with the much more abundant parent species. Consequently, mass spectroscopy is attractive for biomarker discovery as well as routine high-throughput testing.

We agree with Dr. Diamandis that knowing the identities of the proteins comprising the discriminatory ions can potentially lead to insights concerning their sources and relationships to the underlying pathology. In fact, we are using mass spectrometry and enrichment strategies to identify the entire low-molecular-weight region of the proteome. The ions comprising the distinguishing pattern are members of this large unexplored archive. Our findings to date indicate that the low-molecular-weight proteome contains thousands of whole proteins and fragments derived from every class of cellular compartment and ranging from transcription factors to oncogenes to membrane receptors and channels (Mehta et al., submitted for publication; Tirumalai et al., submitted for publication). In the future, we should be able to generate the ion patterns and then go directly to a list of the underlying identities in a database.

Nevertheless, it is our opinion that the clinical evaluation of proteomic patterns should proceed independently from the pursuit of the physiologic sources and identities of these proteins. Indeed, characterization of prostate-specific antigen (PSA) as a cysteine proteinase is not relevant to its utility for prostate cancer screening. CA125 testing was used for many years before we had sequenced and characterized the analyte. As the low-molecular-weight serum proteome becomes fully characterized, serum proteomic pattern analysis could move from ion species to fully identified biomarker molecules and their modified counterparts. However, even now, using highly accurate and precise matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and electrospray ionization (ESI) instrumentation, it is possible to assign a mass to each ion with such precision and accuracy that the accurate mass tag becomes an identifier. In fact, our efforts go beyond the identification and discovery of the ions that comprise the pattern toward means to objectify the ion region around the pattern.

What is needed to validate serum proteomic patterns beyond clinical research study sets to realize routine clinical testing? The critical issues revolve around instrument and process reproducibility and quality control. The Human Proteome Organization, in partnership with the World Health Organization, the American Red Cross, the Food and Drug Administration, and NIST, is developing serum/plasma reference standards. Widespread distribution of standards will be essential for the quality-assurance instruments and the calibration of individual assays. After analyzing many thousands of clinical serum samples, we have encountered many different sources of variability. These findings have emphasized the need for a set of software tools for visualizing and qualifying incoming serum spectral data before diagnostic profiling begins. One successful approach has used statistical profiling of the spectra. We monitor the mean ion amplitude, the sum of ion amplitudes, and the variance of ion species in the low- and high-abundance ranges. Another successful approach uses n-dimensional vector plots calculated from the amplitudes of reference ions that are found consistently throughout all serum samples. Subtle changes in overall amplitude values arising from process variance, which can mask discrimination of the disease state, can be tracked within and between experiments. In-process controls, internal reference standards, release specifications, and stability measures are put in place such that the process can be monitored over a continuous time period.

Other sources of variability and potential bias could arise from differences at the clinic and between clinics. Unbiasing the process is extremely important because subtleties in the way the serum is collected between the cases and controls used for discovery may contribute to artifacts. We have developed standard operating procedures for our clinical collaborators to follow for sample collection, handling, and shipping. Use of the aforementioned quality-control, quality-assurance, and release specifications homogenizes the spectral quality such that when pattern recognition methods are used, the pattern is robust and reflects and is predicated on the disease-state differences. It is imperative that laboratories that are evaluating and investigating pattern diagnostic approaches fully use rigorous spectral quality testing before pattern analysis. Spectral patterns must be identical within the same platform day-to-day, week-to-week, and month-to-month. Moreover, for clinical applications, patterns found on one platform must be identical to those found on another instrument. After our own extensive internal analysis, it is our opinion that research-grade low-resolution mass spectrometry platforms such as the Ciphergen SELDI PBS II or IIc will not be able to consistently deliver the kind of reproducibility required for clinical testing. Our clinical trial will use at least three ABI hybrid quadrupole time-of-flight (QqTof) instruments to assess within- and between-instrument variances in a CLIA- and College of American Pathologists (CAP)-licensed laboratory with a process under design control.

Antibody-based approaches have dominated the clinical chemistry landscape, and mass spectrometry has been used as a clinical analytical method only in specialized areas. Mass spectroscopy platforms of the future, coupled to heuristic pattern recognition algorithms, may become

superior to immunoassays. Current mass spectroscopy platforms have sensitivity in the femtomolar range and will only become more sensitive in the next generation of technology. As mass spectroscopy technology advances, it may be possible to obtain direct biomarker identification "on the fly". We believe that this technology can be most reliable and cost-effective if it is offered through large clinical reference laboratories that have previous experience with sophisticated mass spectroscopy technology. Accordingly, large commercial reference laboratories have undertaken programs to explore mass spectroscopic proteomic patterns for routine diagnosis.

Serum proteomic pattern analysis has already achieved diagnostic sensitivity and specificity superior to those of conventional single biomarkers *(3–8)*. Because of the urgent clinical need for early disease diagnosis, particularly for diseases such as ovarian and pancreatic cancer, we owe it to our patients to rapidly and rigorously test and validate this technology.

References

1. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 2002; 1:845–67.
2. Ward JB Jr, Henderson RE. Identification of needs in biomarker research [Review]. Environ Health Perspect 1996;5(104 Suppl): 895–900.
3. Petricoin EF III, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, et al. Use of proteomic patterns in serum to identify ovarian cancer. Lancet 2002;359:572–7.
4. Petricoin EF III, Liotta LA. Mass spectrometry-based diagnostics: the upcoming revolution in disease detection [Editorial]. Clin Chem 2003;49:533–4.
5. Adam BL, Vlahou A, Semmes OJ, Wright GL Jr. Proteomic approaches to biomarker discovery in prostate and bladder cancers [Review]. Proteomics 2001;1:1264–70.
6. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem 2002;48:1296–304.
7. Petricoin EF 3rd, Ornstein DK, Paweletz CP, Ardekani A, Hackett PS, Hitt BA, et al. Serum proteomic patterns for detection of prostate cancer. J Natl Cancer Inst 2002;94:1576–8.
8. Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, et al. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. Cancer Res 2002;62:3609–14.
9. Chase DH. Mass spectrometry in the clinical laboratory. Chem Rev 2001;101:445–77.
10. Chase, Kalas TA, Naylor EW. The application of tandem mass spectrometry to neonatal screening for inherited disorders of intermediary metabolism. Annu Rev Genomics Hum Genet 2002; 3:17–45.
11. Diamandis EP, Scorilas A, Fracchioli S, Van Gramberen M, De Bruijn H, Henrik A, et al. Human kallikrein 6 (hK6): a new potential serum biomarker for diagnosis and prognosis of ovarian carcinoma. J Clin Oncol 2003;21:1035–43.
12. Kim JH, Skates SJ, Uede T, Wong Kk KK, Schorge JO, Feltmate CM, et al. Osteopontin as a potential diagnostic biomarker for ovarian cancer. JAMA 2002;287:1671–9.