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Introduction: Advances in Protein Analysis for the Clinical Laboratory

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> "The determination of the structure of insulin clearly opens up the way to similar studies on other proteins … One may also hope that studies on proteins may reveal changes that take place in disease and that our efforts may be of more practical use to humanity."
>
> —Frederick S. Sanger, Nobel Lecture
>
> December 11, 1958 (1)

This special issue of *Clinical Chemistry* is devoted to recent developments in the diagnostic application of protein analysis. Proteins have been used as diagnostic markers for disease for more than 150 years— from the development of tests for urinary albumin as an indicator of kidney disease by Bright in 1827 and the first tumor marker, immunoglobulin light chains in urine associated with multiple myeloma, by Bence Jones in 1845. Over the succeeding years, the number of proteins analyzed for diagnostic purposes (2) has gradually increased until, at present, we analyze more than 200 different proteins in serum or plasma, as summarized by Anderson in the present issue (3), and many additional proteins as cellular markers for flow cytometry, red cell antigens, or tissue antigens. The rate of addition of new protein biomarkers has been quite slow, however. Only a few have been added per year, and proteins analyzed for diagnostic purposes represent a small subset of proteins in plasma or the human body. Technological advances allowing simultaneous analysis of hundreds of proteins at a time created hope for identifying a wealth of new protein biomarkers, but progress has been slower than anticipated. Application of multianalyte technologies has identified many candidate biomarkers for disease, but the efforts have been hindered by statistical problems of false discovery; sample stability; dominance of high-abundance components; lack of reproducibility, standardization, and calibration of methods; and lack of sufficient throughput and well-characterized samples for validation of candidate biomarkers (3–7).

In the early 2000s, mass spectrometric analysis showed that serum and plasma contain complex mixtures of small proteins and peptides that have been variously termed the low molecular weight proteome, peptidome, or fragmentome. There was initial enthusiasm and fanfare that profiling of the peptidome offered prospects for diagnosing early-stage cancers (8). Subsequent

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work has not verified initial expectations, and it is apparent that most of the peptides detected are fragments of high abundance proteins (9). Carefully performed clinical trials, such as trials for detection of prostate cancer (10) and for detection of ovarian cancer reported in the present issue (11), fail to identify diagnostic utility for this approach to cancer detection. It is impossible to prove that this approach will never work after further technological improvements, but prospects for peptide profiling as a quick and simple pathway for new cancer tests clearly have waned. Considering that the generation of small peptide fragments in serum and plasma appear to be influenced by proteases released by inflammatory and procoagulant processes, analysis of plasma peptide profiles might be better suited as an approach to diagnose systemic diseases and not for localized cancer.

Although attempts to use peptide profiling for cancer diagnosis have been disappointing, there are many other areas of substantial advance in diagnostic protein analysis. These advances include not only the discovery of new diagnostic markers but also new technological applications; new approaches for multiplex analysis of proteins and interpretation of test results; new methods for analysis of other fluids besides blood, such as cerebrospinal fluid; and improvement of analytical and diagnostic performance of existing tests. Some of the sources of innovation and improvement of clinical laboratory tests are summarized in Table 1. Many of the improvements address practical issues, such as analytical precision, specificity, and sensitivity; throughput and turnaround times; clinical interpretation; and assay standardization. Proteins and peptides have presented particular challenges with respect to calibration and standardization related to structural heterogeneity and instability, and advances in standardization can impact clinical application.

Technological progress in diagnostic testing of proteins is proceeding on several fronts. Robust methods with good accuracy and precision have been developed for the quantitative analysis of proteins and peptides by multiple reaction monitoring mass spectrometry (MRM-MS), [4] in some cases combined with immunoaffinity enrichment at either the peptide or protein level. With the use of MRM-MS, assays have been configured for relatively low abundance components such as thyroglobulin (12), panels of cardiovascular disease–related proteins including troponin I (13,14), and parathyroid hormone, as described in the current issue. Targeted MS approaches offer prospects for measuring particular molecular forms of peptides such as parathyroid hormone and other bioactive peptides with greater specificity. Advances in analytical resolution and mass precision of MS have enabled direct assessment of molecular variation of intact proteins (15). There is continuing progress in the application of planar arrays, bead-based arrays, and new detection technologies to expand the capacity of protein analysis by immunoassays or other specific capture techniques.

A general characteristic of most analyses with good prospects of clinical application is that they are targeted approaches directed at specific peptide or protein components and offer analytical characteristics generally required for clinical laboratory use (5). Challenges presented by multiplex analysis and complex diagnostic algorithms are recognized in a new test classification by the US Food and Drug Administration termed “in vitro diagnostic multivariate index assays” (IVDMIA). Recent clearance by the US Food and Drug Administration of the OVA1^{R} test for assessing risk of ovarian cancer in patients with an ovarian mass serves as one example of an IVDMIA based on analyses of multiple proteins (16).

Identification of new protein biomarkers for disease has been presented as a multistage process having sequential stages of discovery, verification, and validation (7). However, identification,

[4]Nonstandard abbreviations: MRM-MS, multiple reaction monitoring mass spectrometry; IVDMIA, in vitro diagnostic multivariate index assay.

verification, and validation of a new biomarker usually are only preliminary, albeit critical, steps toward developing a new commercially deployable diagnostic test. Generally, analytical platforms used for discovery are not well suited for clinical use. Some of the costs and time barriers to implementation of tests for new protein analytes might be reduced if biomarker research used instrumentation and assays suitable for clinical laboratories. Additional steps in the translation of a new biomarker into a clinical diagnostic test usually include the development of manufacturing processes for reagents, standards, calibrators and controls, engineering and production of new hardware, and development of software. Then, the evaluation for the new test begins over again with evaluation of analytical performance, comparison vs the discovery platform, clinical evaluation, establishing reference ranges and diagnostic cutoffs, and developing control procedures and proficiency-testing programs. Resulting evaluation data must then be submitted for approval by regulatory agencies and for assignment of reimbursement codes. The present issue of *Clinical Chemistry* provides 2 prototypic templates for submissions to the US Food and Drug Administration for multiplex protein-based diagnostic tests, indicating some of the challenges and opportunities with multiparametric panels. The process of test development is iterated many times for a single biomarker as additional manufacturers produce the same test or there is transfer to a new test platform or development of a new generation that improves on the same test. Goals for new versions of a test may include improved sensitivity, specificity, and precision, faster turnaround time, reduced cost, improved automation, and miniaturization to allow performance at the point of care. Consequently, there are many stages in the development and maturation of laboratory tests for a single biomarker. In some cases, changes in analytical performance, turnaround time, cost, and accessibility result in major changes in clinical application of a laboratory test, although the same biomarker is measured. Prominent examples of assays for which generational improvements have led to changes in clinical applications are thyrotropin, C-reactive protein, and cardiac troponins.

Efforts to date, including those described in the current issue, only begin to scratch the surface of the multiple opportunities for diagnostic protein analysis. Most efforts concentrate on measurement of protein concentration. Use of a more sophisticated instrument, such as a mass spectrometer, to measure abundance of a tryptic peptide does not necessarily constitute a fundamental advance over the use of an antibody to measure epitope abundance. However, the emerging picture of improved molecular specificity, isoform discrimination, true internal standardization with isotopically labeled peptides, and simultaneous analysis of multiple components makes a compelling case for some applications of MRM-MS. The ability to perform multiplex analyses offers new opportunities for internal standardization via ratioing of components and for application of complex diagnostic algorithms, as recognized by the IVDMIA classification, rather than simply evaluating a test result vs a reference range or cutoff value. There sometimes is an unreasonable expectation that measuring protein concentration at a single time point can provide a diagnosis. The example of markers for myocardial infarction, however, demonstrates the value of serial sampling that may be more widely applied with temporal responses to physiological stimuli or with measurements over longer time intervals as long as months or years to examine temporal changes.

The vision of Frederick Sanger expressed in his 1958 Nobel address (1), to identify protein changes in disease, has been only partially realized. The diagnostic possibilities represented by the complex array of covalent and conformational states of proteins as well as their occurrence in macromolecular complexes and interaction networks is only beginning to be explored. Therefore, development of tests for specific measurements of protein structure and interactions offers considerable unexplored potential. It is clear that there remain many opportunities for applying new biomarkers, new technologies, and refinements of existing tests to diagnostic applications of protein analysis. The present issue of *Clinical Chemistry* describes

just a few of the many possibilities presented by analysis of the more than 20 000 gene products and their multiple structural variations and complex molecular interactions.

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Table 1

Sources of innovation and improvement in protein diagnostics.

• Biomarker discovery, verification, and validation	
• Application of new analytical technologies	Mass spectrometry
	Multiplex immunoassay
	New detection methodologies
	Methods for protein fractionation
• Platform engineering	Automation
	Analysis time
	Miniaturization
• Reagent development	New capture reagents
	New labeling reagents
	Stabilization of reagents
• Interpretation	New clinical data
	New diagnostic algorithns
• Preanalytical processes	Patient preparation
	Specimen collection and processing
• Standardization	Development of reference materials
	Development of reference methods
• QC and proficiency testing	