Protein Quantitation through Targeted Mass Spectrometry: The Way Out of Biomarker Purgatory?

The enormous potential of biomarkers to revolutionize clinical practice and improve patient care has been well documented (1, 2). Molecular-based diagnostic and prognostic tests, particularly those aimed at protein analytes, could be used to detect disease earlier, enabling treatment to start sooner and possibly cure rather than to merely delay further injury or death. These tools could also be used to stage disease more accurately and to predict response to therapy, thereby helping to select the correct treatment. Biomarkers can also be used to stratify patients for the assessment of new drug therapies and to serve as surrogate endpoints in early-phase drug trials, thereby lowering the overall cost of drug development and producing more effective treatments. Given their high potential therapeutic and financial impacts, that so few new protein biomarkers have been introduced into widespread clinical use recently is, on the surface, surprising. In fact, only 5 new protein markers have been approved by the US Food and Drug Administration in the last 5 years for measurement in plasma or serum [the information on protein markers in Anderson and Anderson, 2002, has been updated with information from the Center for Devices and Radiological Health, US Food and Drug Administration] (3, 4).

The reasons for the dearth of new protein biomarkers are gradually becoming clearer. They are related to the high false-discovery rate of "omics" methods (regardless of the technology used), combined with a lack of robust methods for biomarker verification in large clinical sample sets (5–8). It is now common for differential analyses of tissue or plasma samples by multidimensional liquid chromatography–tandem mass spectrometry (LC-MS/MS)[1] (the workhorse tool for unbiased discovery) to confidently identify thousands of proteins, hundreds of which can vary in concentration by 5-fold or more between case and control samples in small discovery studies. To access proteins at lower abundances (e.g., <500 μg/L in plasma, concentrations at which occur many of the known protein biomarkers, such as carcinoembryonic antigen, pros-

tate-specific antigen, neuron-specific enolase, and the troponins), these studies always employ multidimensional fractionation at the protein and/or peptide level, thereby exploding a single patient sample into up to 100 subfractions, each requiring lengthy LC-MS/MS analysis. It is not uncommon for the analysis of a single case/control sample pair to take up to 2 weeks of on-instrument time, which limits the numbers of samples that can be practically analyzed to typically 10 (or fewer) case–control comparisons. These numbers are very small relative to the high dimensionality of the proteome (hundreds of thousands or more possible components, when posttranslational modifications and other variants are taken into account) and the scale of typical variation in the human population. Thus, a very large fraction, possibly exceeding 95%, of the protein biomarkers "discovered" in these experiments are false positives that arise from biological or technical variability. Clearly, discovery "omics" experiments do not lead to biomarkers of immediate clinical utility, but rather produce "candidates" that must be "qualified" and "verified" (7, 8).

Until recently, verification technologies capable of testing large numbers of protein biomarker candidates emerging from discovery "omics" experiments in large sample sets (>1000–2000) have not been available. In principle, antibody (Ab)-based measurements could be used; however, the required immunoassay-grade Ab pairs exist for only a small number of the potential candidate biomarker proteins. Developing a new, clinically deployable immunoassay is both very expensive (US $100 000 to $250 000 per biomarker candidate for a research assay, or $2–4 million for a Food and Drug Administration–approvable assay) and time-consuming (1–1.5 years). This fact restricts the use of immunoassays to the short list of already highly credentialed candidates. For the large majority of new, unproven candidate biomarkers, what is required is an intermediate verification technology with shorter assay-development time lines, lower assay costs, effective multiplexing of 10–50 candidates, low sample consumption, and a high-throughput capability for analyzing hundreds to thousands of serum or plasma samples with good precision. The goal of such a verification approach would be to identify from the initial list of hundreds of candidate protein biomarkers the few that are worth advancing to traditional candidate-validation

---

[1] Nonstandard abbreviations: LC-MS/MS, liquid chromatography–tandem mass spectrometry; Ab, antibody; SID-MRM-MS, stable-isotope–dilution multiple reaction–monitoring mass spectrometry; LOQ, limit of quantification; SISCAPA, stable isotope standards with capture by antipeptide antibodies.

Editorial

studies that use assays deployable on clinically approved analysis platforms.

The core technology that has emerged for verification of candidate biomarkers is stable-isotope–dilution multiple reaction–monitoring mass spectrometry (SID-MRM-MS) (9, 10), an approach that has been used very successfully for quantifying small molecules (e.g., hormones, drugs, and their metabolites) in pharmaceutical research and, more recently, in clinical laboratories. The use of SID-MRM-MS for protein assays is predicated on the measurement of "signature" or "proteotypic" tryptic peptides that uniquely and stoichiometrically represent the protein candidates of interest. MRM-based assay development starts with the selection of 3–5 peptides per protein (10). Synthetic, stable isotope–labeled versions of each peptide are used as internal standards, enabling protein concentration to be measured by comparing the signals from the exogenous labeled and endogenous unlabeled species. Peptide selection is driven by the initial discovery data as well as by additional experiments, such as accurate inclusion mass screening (10), and information available in on-line databases, such as the Global Proteome Machine (GPM) (12) and PeptideAtlas (13), that identify peptides that have been observed in previous proteomics experiments. Response curves are obtained for each peptide in the matrix of trypsin-digested plasma to evaluate potential interferences and to establish the limit of quantification (LOQ) and the limit of detection for each peptide. One to 2 configured assays are produced for any given protein.

SID-MRM-MS assays have several features that distinguish them from conventional immunoassays. First, the analyte detected in the mass spectrometer can be characterized with near-absolute structural specificity—something never possible with antibodies alone. This characteristic is a potentially critical quality advantage, especially in cases in which immunoassays are subject to interferences. Second, MRM assays can be highly multiplexed such that 20 or more proteins can be measured during a single analysis (9, 10), with assay CVs of <10% having been demonstrated for proteins at concentrations ≥1 mg/L in plasma (9). Third, all of these measurements can be done with approximately 100 nL of plasma. In contrast, individual immunoassays often consume 10–100 μL of plasma (i.e., 100–1000 times as much).

Many biomarkers of current clinical importance, such as prostate-specific antigen, carcinoembryonic antigen, and the troponins, reside in the tens-of-nanograms-per-liter to the low-micrograms-per-liter range in plasma. Are SID-MRM-MS methods capable of such sensitivity? Keshishian et al. have recently shown that a combination of depletion of abundant proteins and minimal fractionation of tryptic peptides by strong cat-

ion exchange before SID-MRM-MS provides LOQs in the range of 1–20 μg/L with CVs of 10%–20% at these LOQs for these proteins in plasma (10); however, such extensive sample processing restricts sample throughput substantially compared with immunoassays. Detection of proteins in the mid-to-low nanogram-per-liter range is not currently possible with this approach because of the current limits of mass spectrometer sensitivity. Improvements in instrument design anticipated in the near future may help break this analytical barrier [for example, see (14, 15)].

An approach known as stable isotope standards with capture by antipeptide antibodies (SISCAPA) combines the advantages of specific immunoaffinity enrichment of a target peptide with the structural specificity and quantitative capabilities of SID-MRM-MS (16, 17). In this approach, antipeptide antibodies are made against the selected signature tryptic peptides from the proteins of interest. After tryptic digestion of the plasma and addition of known amounts of stable isotope–labeled calibrator peptide, both added and sample-derived versions are specifically enriched, and the relative amounts are measured by MRM. Although the affinity of the Ab for the peptide must be quite good, requirements for selectivity can be relaxed because the mass spectrometer is capable of specifically detecting and quantifying the signature peptides, even in the presence of a highly complex background. Recent studies suggest that enrichment of >1000-fold can be achieved with this approach for plasma-digest peptides (17) and that SISCAPA assays can achieve LOQs in plasma in the low microgram-per-liter range with CVs of <20%. In substituting one Ab affinity step at the peptide level for more complex multistep sample-fractionation schemes, SISCAPA improves throughput (e.g., in the magnetic bead format) while likely permitting at least 10 assays to be multiplexed.

The report by Hoofnagle and coworkers in this issue of *Clinical Chemistry* is another important contribution to the emerging SID-MRM-MS and SISCAPA literature (18). These investigators used a polyclonal antipeptide Ab to develop the first SISCAPA assay implemented in a clinical laboratory environment—an assay for serum thyroglobulin, an established tumor marker whose existing immunoassays are plagued by frequent interferences that negatively affect clinical performance (19, 20). The authors demonstrate LOQs in the low microgram-per-liter range and acceptable assay CVs that are consistent with the sensitivity and assay CVs reported for other MRM (10) and SISCAPA (16, 17) assays, and the results correlate well with current immunoassay results. The work provides a fine example of how MRM and SISCAPA assays can be readily configured for new target proteins and how these assays avoid many of the common problems as-

sociated with immunoassays when the analyte is in the current detection range of >1 µg/L in blood. Through the use of peptide (as opposed to protein) immunoaffinity enrichment before SID-MRM-MS, the authors avoid potential interferences with endogenous immunoglobulins that are commonly encountered in immunoassays (such interfering components being digested to noninteracting peptides before the specific capture of target peptides). The attractive notion that SISCAPA assays will be entirely interference free is probably not correct, however. Although these assays will likely not suffer from host Ab interference, they can be subject to interference by peptides having nearly the same epitope or the same epitope in a slightly different sequence context that are still recognized and captured by the Ab. Nevertheless, unlike immunoassay measurements, the presence of an interference will become immediately clear, provided the mass of the interfering peptide is even just a few daltons different from that of the target peptide.

Is quantitative mass spectrometry of peptides ready to follow MRM of steroids and immunosuppressants into the clinical laboratory? MRM methods coupled with SISCAPA have the potential to produce results of sufficient sensitivity, reproducibility, and ruggedness for eventual adoption into clinical laboratories. Such methods already have the distinct advantages over ELISA methods of enabling rapid development of new assays at relatively low costs while retaining the ability to produce results of very high quality. SISCAPA-MRM methods also have unique advantages (relative to ELISAs), such as definitive characterization of analyte structure, facile detection of interferences, and ease of multiplexing. This tremendous promise may eventually be realized in the clinical laboratory if future studies demonstrate good intra- and interlaboratory assay reproducibility across a wide range of protein analytes and if assay sensitivity can be further improved. It is hoped that compelling and repeated demonstration of SISCAPA-MRM capabilities will lead instrument vendors to develop facilities for fully automated and reliable sample preparation, LC-MS/MS instruments for routine clinical laboratory use, and a full menu of assays for known protein biomarkers. Such developments would spur radical improvements in biomarker verification and validation and provide a far smoother path from biomarker discovery to clinical implementation than exists today.

– **Steven A. Carr**[2]*
– **Leigh Anderson**[3]*

[2] *Broad Institute of MIT and Harvard*
   Cambridge, MA
[3] *The Plasma Proteome Institute*
   Washington, DC
* *Address correspondence to these authors at:*
   Steven A. Carr
   Broad Institute of MIT and Harvard
   7 Cambridge Center
   Cambridge, MA 02142
   Fax 01-617-252-1902
   E-mail scarr@broad.mit.edu;
   Leigh Anderson
   Plasma Proteome Institute
   P.O. Box 3450
   Washington, DC 20009
   E-mail leighanderson@plasmaproteome.org

References

1. Etzioni R, Urban N, Ramsey S, McIntosh M, Schwartz S, Reid B, et al. The case for early detection. Nat Rev Cancer 2003;3:243–52.
2. Trusheim MR, Berndt ER, Douglas FL. Stratified medicine: strategic and economic implications of combining drugs and clinical biomarkers. Nature Rev Drug Disc 2007;6:287–93.
3. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 2002;1:845–67.
4. Center for Devices and Radiological Health, US Food and Drug Administration. Clinical Laboratory Improvement Amendments—Download Data. http://www.fda.gov/cdrh/clia/downloaddata.html (Accessed August 2008).
5. Anderson NL. The roles of multiple proteomic platforms in a pipeline for new diagnostics. Mol Cell Proteomics 2005;4:1441–4.
6. Gutman S, Kessler LG. The US Food and Drug Administration perspective on cancer biomarker development. Nat Rev Cancer 2006;6:565–71.
7. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat Biotechnol 2006;24:971–83.
8. Paulovich AG, Whiteaker JR, Hoofnagle AN, Wang P. The interface between biomarker discovery and clinical validation: the tar pit of the protein biomarker pipeline. Proteomics Clin Appl Forthcoming 2008.
9. Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Mol Cell Proteomics 2006;5:573–88.

10. Keshishian H, Addona T, Burgess M, Kuhn E, Carr SA. Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. Mol Cell Proteomics 2007;6:2212–29.

11. Jaffe JD, Keshishian H, Chang B, Addona TA, Gillette MA, Carr SA. Accurate inclusion mass screening: a bridge from unbiased discovery to targeted assay development for biomarker verification. Mol Cell Proteomics 2008;7:1952–62.

12. Craig R, Cortens JP, Beavis RC. An open source system for analyzing, validating and storing protein identification data. J Proteome Res 2004;3:1234–42.

13. Desiere F, Deutsch EW, King NL, Nesvizhskii AI, Mallick P, Eng J, et al. The PeptideAtlas project. Nucleic Acids Res 2006;34:D655–8.

14. Kelly RT, Page JS, Zhao R, Qian W-J, Mottaz HM, Tang K, Smith RD. Capillary-based multi nanoelectrospray emitters: improvements in ion transmission efficiency and implementation with capillary reversed-phase LC-ESI-MS. Anal Chem 2008;80:143–9.

15. Page JS, Tang K, Kelly RT, Smith RD. Subambient pressure ionization with nanoelectrospray source and interface for improved sensitivity in mass spectrometry. Anal Chem 2008;80:1800–5.

16. Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). J Proteome Res 2004;3:235–44.

17. Whiteaker JR, Zhao L, Zhang HY, Feng LC, Piening BD, Anderson L, Paulovich AG. Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers. Anal Biochem 2007;362:44–54.

18. Hoofnagle AN, Becker JO, Wener MH, Heinecke JW. Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. Clin Chem 2008;54:1796–1804.

19. Spencer CA, Takeuchi M, Kazarosyan M, Wang CC, Guttler RB, Singer PA, et al. Serum thyroglobulin autoantibodies: prevalence, influence on serum thyroglobulin measurement, and prognostic significance in patients with differentiated thyroid carcinoma. J Clin Endocrinol Metab 1998;83:1121–7.

20. Hoofnagle AN, Wener MW. Serum thyroglobulin: a model of immunoassay imperfection. Clin Lab Int 2006;8:12–4.