

Host: This is the podcast from *Clinical Chemistry*. I am Bob Barrett.

Remember the word urgently whispered into Dustin Hoffman's ear, in the 1967 movie *The Graduate*, that secret word that would lead him on to a great fortune? Of course you do: "plastics!" At the World Economic Forum in Davos, Switzerland, the word for 2001, the linguistic key to the future was whispered into a reporter's ear by a great scientist: "proteomics." That's how William Safire opened his "On Language" column in *the New York Times* in February 2001, and over the past decade, proteomics has firmly established itself in clinical research.

Proteins have been analyzed for diagnostic purposes for more than a century. Recent advances in protein analysis have expanded the opportunities for quantitative analysis of proteins and the detection of molecular variants resulting from genetic variation and post-translational modifications. This has created the potential for new diagnostic applications and some of the new opportunities in protein analysis are described in a special issue of *Clinical Chemistry* published in February.

We are fortunate to have with us today the editors of the Proteomic Special Issue.

Dr. Glen Hortin is a Clinical Professor in the Department of Pathology at the University of Florida, College of Medicine. Steven Carr is a senior scientific leader in protein biochemistry and proteomics and leads the proteomics platform at the Broad Institute of MIT and Harvard. And Dr. Leigh Anderson is Founder and CEO of the Plasma Proteome Institute in Washington, D.C.

Gentleman, William Safire also said that the proper term should be "proteinomics." Dr. Hortin, would you agree? What exactly is proteomics?

Dr. Glen Hortin: Technically, proteomics refers to studying the complete set of proteins. In practice, many people have started using it to refer to analysis of sets of perhaps hundreds of proteins, and that's become a little bit confusing, I think. Really we are kind of in a situation where the term has become a little bit confusing in application.

Host: Dr. Anderson, would you like to add to that?

Dr. Leigh Anderson: Yeah, I think proteomics is a term that's important for framing what people are doing at this point, but it's very limiting in certain senses. Clearly, at this stage, we're just beginning to learn how much diagnostic value can be obtained from studies of the amounts of specific proteins,

which most protein-based chemical tests are today. And then how much more can be learned by studying post-translational modifications, splice variants, cleavage or protein etcetera.

So, this is an important sense sort of terra incognita, and it's clearly much more complex than the genome has been. I think the genome is, in the sense, arrived at the right time for proteomics, because it allows us to organize the discovery and provides a huge foundation for the technologies that are used.

But at this point, given the complexities of the proteome and the heterogeneity of methods that are used to approach it, it's a field that is so large that it doesn't really fit completely under that word "proteomics" anymore, and some people have suggested that we should just concentrate on the measurement technologies and on the biology and not think so much of proteomics as a circumscribed field onto itself.

Host: And Dr. Carr, how do you define proteomics?

Dr. Steven Carr: Well, I think, both Glen and Leigh did a very good job. I would add to that that you do need to have a kind of a term, which allows people to get in their heads a conception of what it is that you are talking about. I think "proteomics" does a reasonable job of that. However, as Leigh pointed out, it really is kind of a catchall term. It started out as really the analysis of anything more than two proteins at a time, which considered to be initially proteomics, but it's really become much, much more complex than that.

We are now capable of analyzing, I would say, on the order of thousands of proteins from tissue samples or clinical materials or cultured cell lines, and we are able to do this now using strategies that not only detect what the peptides are that come from these proteins, because remember that proteomics is fundamentally at least, as practiced today, still detect peptides and roll them up to the identities of the proteins.

So, these strategies not only detect the peptides and therefore the proteins, but they're also able to quantify the expression levels from the modification states, and the latter is particularly important, because there really is no other technology out there which allows one to, say, analyze the phosphorylation or glycosylation, or lipidation state of proteins. And we do this now in states of health and disease, in the presence or absence of a drug treatment as a function of development, etcetera. So, that to me is proteomics.

So, it's not just all about human samples, of course, because much of modern biology today is still practiced using model organisms, so the fact that we can do these studies in systems like *C. elegans* or yeast or in animal models like the mouse is all under proteomics umbrella as well, and none of it would be possible without having the genome.

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Host: With that in mind, Dr. Hortin, have there been any new clinical laboratory tests that have resulted from research in proteomics?

Dr. Glen Hortin: Well, the OVA1 Test is kind of one recent example of a test that was cleared by the FDA resulted from discovery efforts kind of applying techniques that some people would classify as proteomic techniques, such as this, a test that really tries to help determine of an ovarian mass, represents ovarian cancer. I think there are quite a few other tests on horizon, and a lot of new opportunities are opening up. It's hard to know exactly which test will arise from these. Ones that seem to be in terms of most rapidly available, probably would be analysis of bioactive peptides where many of these mass spectrometric methods can be used to measure these directly. I think that will open up, to some degree, quite a few new assays in terms of measuring bioactive peptides.

Host: And what new clinical laboratory tests have you seen, Dr. Carr?

Dr. Steven Carr: Really, none beyond what Glen has mentioned. I think we are still in the realm, preclinical application of discoveries coming out of proteomics. I think in terms of applications of new tests that leverage mass spectrometry-based methods, I think if I could frame the question that way, I would say the answer is there are new things coming along, that I would point to Andy Hoofnagle's thyroglobulin assay, which is a MS-based assay using targeted mass spectrometry-based methods that overcomes many of the limitations of immunoassay methods that are currently available.

Host: And Dr. Anderson, anything you'd like to add?

Dr. Leigh Anderson: Well, I agree with what Glen and Steve have framed as the situation there. It's striking that out of little over 200 protein-based assays that are available to physicians in this country, none of them except this new OVA1 Test with a very limited indication, is arising from proteomics. This is a question that has gotten a lot of discussion, and it should be on the tops of the mind of the people in this field I think.

The general conclusion I believe at the moment is that the key impediment to realizing the kinds of benefits that could come from a flow of new candidate protein markers from proteomics really has to do with the lack of the downstream components of a pipeline to move what are effectively discoveries of candidate markers that come out of proteomics through some kind of extensive shifting process to verify that they really have clinical significance and therefore, make them attractive to the clinical diagnostic community, both the commercial side and the clinical laboratorian to implement them as test.

This is something that is in some sense are structural as well as technical problem in this field, one which I think is showing an immense amount of progress, and the prospects appear to be very good for a substantial increase in the flow of these candidates into clinical medicine in the near future.

Host: Okay. Now Dr. Carr, what are some of the major new areas of opportunity in protein analysis?

Dr. Steven Carr: Oh my goodness. How much time do we have? I think there are really many new opportunities exist. They are being made possible by rapid advances in mass spectrometry technologies that really continue unabated. They are also being made possible by the speed and accuracy of the data processing software, that's now routinely available and robust and also being facilitated by the endless creativity of scientists in the proteomics field, who are really continually developing new and better methods for preparing samples for MS analysis.

I think you can break these opportunities down into various applications, and I will, but I want to start by emphasizing that all of the studies that I'll mention can now be done with precise relative quantification using either metabolic or chemical labeling or external standards, labeled external standards, and they are done routinely in cultured cell lines through animal models and human clinical samples. And using these labeling strategies has really facilitated our ability to detect even relatively small changes in protein expression levels between samples in a temporal fashion in states of disease and development and drug dose.

So with respect to application, I start with clinical applications, and this sort of follows on from where Leigh left off answering the previous question. But I think these opportunities are being driven by the recent developments in the application of selective reaction monitoring or multiple reaction monitoring as it's also referred to mass spectrometry, which are targeted analyses of proteins really peptides from proteins in complex matrices like plasma or tissue, although the methods certainly aren't limited to that,

and they are becoming widely used in biological studies and model organisms, such as yeast as well.

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When you combine these approaches with affinity enrichment that either the protein at the peptide level, which really enables rapid high throughput quantitative analysis of proteins present at fairly low abundance, and we are talking here at the bottom of the nanogram/mL plasma and even into the pictogram/mL plasma. There have been tests configured now for clinically deployed markers like PSA and thyroglobulin in the components, but more importantly these technologies are proving to be capable of serving as a bridge between the discovery proteomics methods, which Leigh mentioned, in which there is lots and lots of discoveries emerging from, but unfortunately very few of them are entering into actual clinical implementation.

So, you need something to bridge that and immunoassays presently cannot be used for that because the quantity and the quality of such assays is not sufficient to address the need. MRM has this potential to be used as an assay drafting tool for configuring assays that have sufficient performance for being able to quantitatively measure potential markers and hundreds of patient samples and really build the credentialing around them.

The instruments that are being used through for these applications are also experiencing very rapid improvements and sensitivity and specificity. I'll just mention one and that's ion mobility mass spectrometry, and this is the method that enables the separation of ions at different charge state and confirmation on millisecond timescales in-line with the mass spectrometer. You can think of it as another level of chromatographic separation without any sacrifice in the analysis speed, and it's likely that advances like this will lead to tenfold or higher levels of improvements in sensitivity and specificity in these analyses in the near future.

Another significant area of opportunity is in post-translational modification analysis. Mass spectrometry has always been the go-to technology for PTM characterization, but revolutionary, not just evolutionary, but revolutionary improvements in mass spectrometry, mass accuracy, and the resolution and the speed at which sequencing can be done on these instruments together with improved sample handling methods like affinity-enrichment techniques have enabled previously impossible analyses to be done.

For example, we can now easily do proteome-wide analysis of phosphorylation and acetylation, and this is not just to

pick up of a few hundred phosphosites, we now are routinely getting 5,000 to 6,000 phosphosites when we characterize State A versus State B comparisons, and again done quantitatively.

Finally, there are new advances in ion activation which are making all sorts of new opportunities possible. There is a technique called "electron transfer dissociation," which now makes it possible to analyze large pieces of proteins not just small peptides without the loss of labile modifications. So, with ETD, for example, it's now possible to easily analyze the N-terminal shift in the amino acids of histones, and to identify in the same molecule the entire panoply of modifications that exist on that protein.

It's also sparking advances in top-down proteomics for analysis of entire proteins. Again, I could go on, imaging MS is still making rapid improvements particularly for tracking the presence and location of small molecules and tissues, and I think all of the improvements in sensitivity are going to drive the application of these methods toward clinically derived materials, for example, punch biopsies, which presently are relatively inaccessible for analysis, simply because of the amount of protein is too small.

Host: Dr. Hortin, what are your thoughts?

Dr. Glen Hortin: I think Steve covered a lot of areas, I'd just add a couple of other areas. In terms of the clinical laboratory, an important area is the immunoassay field, the development of multiplex immunoassays either in planar arrays where multiple antibodies are placed at different spots at different locations within a planar surface or as beta rays in which different antibodies are attached to different beads and separated basically in a flow cytometer.

So those are actually tools that are coming in to fairly routine use and are available on some clinical platforms. At the present time the level of multiplexity on those for the clinical lab is a little bit lower, perhaps up to—maybe limited to about 10 or 20 assays at a time. I'm not looking at real large numbers, but I think these technologies come in along in a variety of other technologies for multiplex immunoassays, they will probably allow some expansion in terms of the number of assays that can be formed at the same time, and that allows you to minimize the amount of the sample that you need by basically doing multiple analysis on a single sample aliquot, and of course multiples the amount of information available.

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Other areas I think that Steve discussed a little bit in terms of kind of the top-down proteomics, which is basically analysis by mass spectrometry of an intact protein, that allows you to really examine the molecular variation among different individuals and that has been enabled really to some degree by the advances in the resolution of the mass spectrometry that allow you high enough resolution to pick up very tiny mass changes in an intact protein molecule, and that provide in a lot of new information, I think in terms of molecular variation and the post-translational modifications of molecules.

And particularly if you move to a little bit smaller molecules, such as bioactive peptides, that distinction and ability to distinguish a minor post-translational modification or a difference of one or two amino acids in length, often times it's critical for the bioactivity of the peptide. So, it allows you to distinguish the bioactive forms from the nonbioactive forms, which may be difficult to distinguish by immunoassay. So those were just a couple of areas that I would add to what Steve mentioned.

Host: And Dr. Anderson, anything you would like to add.

Dr. Leigh Anderson: I think the previous answers cover a lot of the particular kind of questions that would be opened up by advances in the technology and the science going forward, and I think that's a very good summary. So, I would like to just point out one additional area in which effort, I think, is going to be extremely well rewarded from a biological insight point of view and it's particularly related to the clinical laboratory, and that's the study of populations.

One of the key features of good protein biomarkers is that they behave similarly in a disease state across individuals in a population, and typically in proteomics and biomarker work, this question of how variable proteins are or post-translational modifications, for that matter across the population, is only addressed very late in the series of investigation that are pursued to try to verify the use of a new biomarker.

This is something which can easily be done much earlier in the process and on a much more systematic basis through clinical laboratories. Clinical laboratories had access to the assays for candidate biomarkers for example. It would be relatively straightforward to collect the information on their performance in population and begin to get a much more general and scientifically much more satisfactory view of the variation in protein expression, protein modification across populations of interesting patients. This is something that would be enabled if the technologies that we are talking about can gradually penetrate the clinical laboratory. There

are a wide range of biologically extremely informative studies that can be done on large sets of even the identified samples that flow through clinical labs.

Host: Well, Dr. Anderson, in your opinion, how will new advances in protein analysis affect the clinical laboratory?

Dr. Leigh Anderson: I think this is an open question at this point. Clearly, we have been talking so far about the changes that would follow the introduction of useful, new clinical tests, and I believe that new tests plus the realization that in many cases, the patient is by far his or her own best control, and therefore longitudinal test results are important. And finally, the realization that in many cases of a multiplex test involving a series of analytes interpreted together to give one result.

All of these things conspired to, in the long run, increase the amount of analytical work which will be done in clinical laboratories very substantially. In fact, in terms of assay volume, I think if these things come to pass, as we currently expect them to, there should be a doubling or up to a tenfold increase in the number of results in the protein space that are going to be required for clinical laboratories going forward, and obviously technology improvements and cost reductions are going to be necessary to make this possible.

But I think that's sort of a challenge side of it. There is an upside to this as well, which is that some of us feel that it has not, I'll confess not yet, really demonstrated that some of the methods, particularly, involving mass spectrometry, which has much higher specificity than can be achieved with immunoassays and allow for direct internal standardization within assays. If these methods can in fact be made to be as accurate and as sensitive as current immunoassay and ultimately multiplexible at lower cost, the opportunity exists to move an entire new technology platform, at least, at the protein levels in the clinical laboratories to measure proteins.

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Now this isn't brand-new in the clinical lab because mass spectrometry is used to measure small molecules like steroids and immunosuppressants in clinical laboratories now, but if there is, in fact, the opportunity to develop platforms that work, that are sufficiently robust to work in clinical labs based on technologies like these, then it's quite possible that a bridge of common technology can be established between the biomarker community and the clinical laboratories that could cut literally years if not portion of a decade off of the time required to transition new assays in the clinical labs, and at the same time enable

much closer collaboration between the biomarker discovery and verification communities and clinical laboratories.

That would be an extremely positive development from my point of view to see if it can be realized in the near term.

Host: And Dr. Hortin, your thoughts?

Dr. Glen Hortin: I think actually the current advances are going to have a major impact in the routine clinical laboratories, and some of this may show up really in the forms of better standardization or restandardization of assays. I think that the example of hemoglobin A1c is one current example where the recent development of mass spectrometric assays that provide a greater specificity led to some readjustment of the values that are being applied to hemoglobin A1c values.

So even for assays that we have used, traditional methods that they may be immunoassay methods or in the case of hemoglobin A1c, there may be a chromatographic methods. The new mass spectrometric method, the greatest specificity allows us to actually measure with greater specificity the molecules that we want to and determine the true value, and I think that it is providing a much better understanding of what we are measuring by some of the immunoassays. Take the example of the troponin assays where there is lots of confusion now, and lack of agreement between different assays. I think over the next couple of years and really a work in progress now is sorting out what the different assays are measuring and why they do not provide equivalent results.

So, this basic understanding will really allow us to apply even routine immunoassays, and we may result in restandardization of some of these. Down the road I think the ability to increasingly analyze molecular variants approaches will open up quite a few new assays. There has been some discussion about the ability to do multiplex assays are going to substantially increase the amount of information that we have. So those are the main points that I'd cover.

Host: Well, then we will move on to our final question. Tell us exactly what is so special about this special issue of *Clinical Chemistry* on proteomics and what should readers of the print issue expect, Dr. Hortin?

Dr. Glen Hortin: Well, I think in the field that there are really large, a numbers of different areas that are moving forward very rapidly now. So, I think the special issue really provides, to some degree, a sampling of different areas ranging from advances in immunoassay techniques to mass spectrometric applications.

That's one aspect of it just to try to get some feel for where fields are moving forward. I think the other thing is that really to some degree what is special about the issue is to try to start to address all of these things can be applied in the clinical laboratory, and I think there are a number of points and discussion and materials in the special issue, which address issues in terms of regulatory issues and other practical issues that need to be addressed in terms of moving forward and kind of bringing the assays into the clinical lab.

Host: Dr. Carr?

Dr. Steven Carr: I think what's special about it is that it's not just about proteomics, it really is the focused application of proteomics in the clinical problems of interest, and toward that end there are articles in here covering in a review fashion where we are presently in the application of mass spectrometry-based biomarker discovery in cardiovascular disease, what the needs are, and what the opportunities are.

As Glen mentioned there are articles that focus on the challenges of moving assays into true clinical implementation both multiplexed immunoassay platforms, novel platforms, as well as targeted MS-based methods. So there are a number of papers in the journal that focus on those questions. There are a number of papers which deal with new applications of targeted mass spectrometry methods for detection of selected proteins, some of them in a multiplexed fashion in blood and other biological fluids. So again, highlighting what the current state of the art in application of these methods is.

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That combination of articles really is what makes this special. It's touching on many different aspects of not just mass spectrometry, but other protein analysis methods in the clinical setting and what the opportunities are and where we presently are in those various fields.

Host: Dr. Anderson, you get the last word today.

Dr. Leigh Anderson: What satisfies me I think particularly about this special issue is that in some ways it marks a progression past a period where many people were very hopeful and enthusiastic about the contribution proteomics can make, and a few well-known cases, some hype was generated along with the hope, but at this stage people, I believe, have confronted the major problem that exists and what many of the authors of the papers in the special issue have to say is infused with

a really realistic sense of where we are and how much farther we have got to go and what the challenges are.

One example of that I find very encouraging is that people in the regulatory agencies, particularly the FDA, participated directly in some of these papers, and there is sort of a legitimate community spirit evolving with respect to how we are going to confront the very difficult problems of finding which really are the right biomarkers and getting them really into clinical practice.

I don't think there are any major parts of the problem that are left out of this issue, and that I think gives it some real unique character in the history of publication in this field. So it should very useful I think to the clinical chemistry community.

Host: Gentlemen, thank you so much for your time today.

Dr. Leigh Anderson: You are welcome. Thank you.

Host: Dr. Glen Hortin is a Clinical Professor in the Department of Pathology at the University of Florida, College of Medicine. Steven Carr is a senior scientific leader in protein biochemistry and proteomics and leads the proteomics platform at the Broad Institute of MIT and Harvard. And Dr. Leigh Anderson is Founder and CEO of the Plasma Proteome Institute in Washington, D.C.

They have been our guests in this podcast from *Clinical Chemistry*. I am Bob Barrett. Thanks for listening.