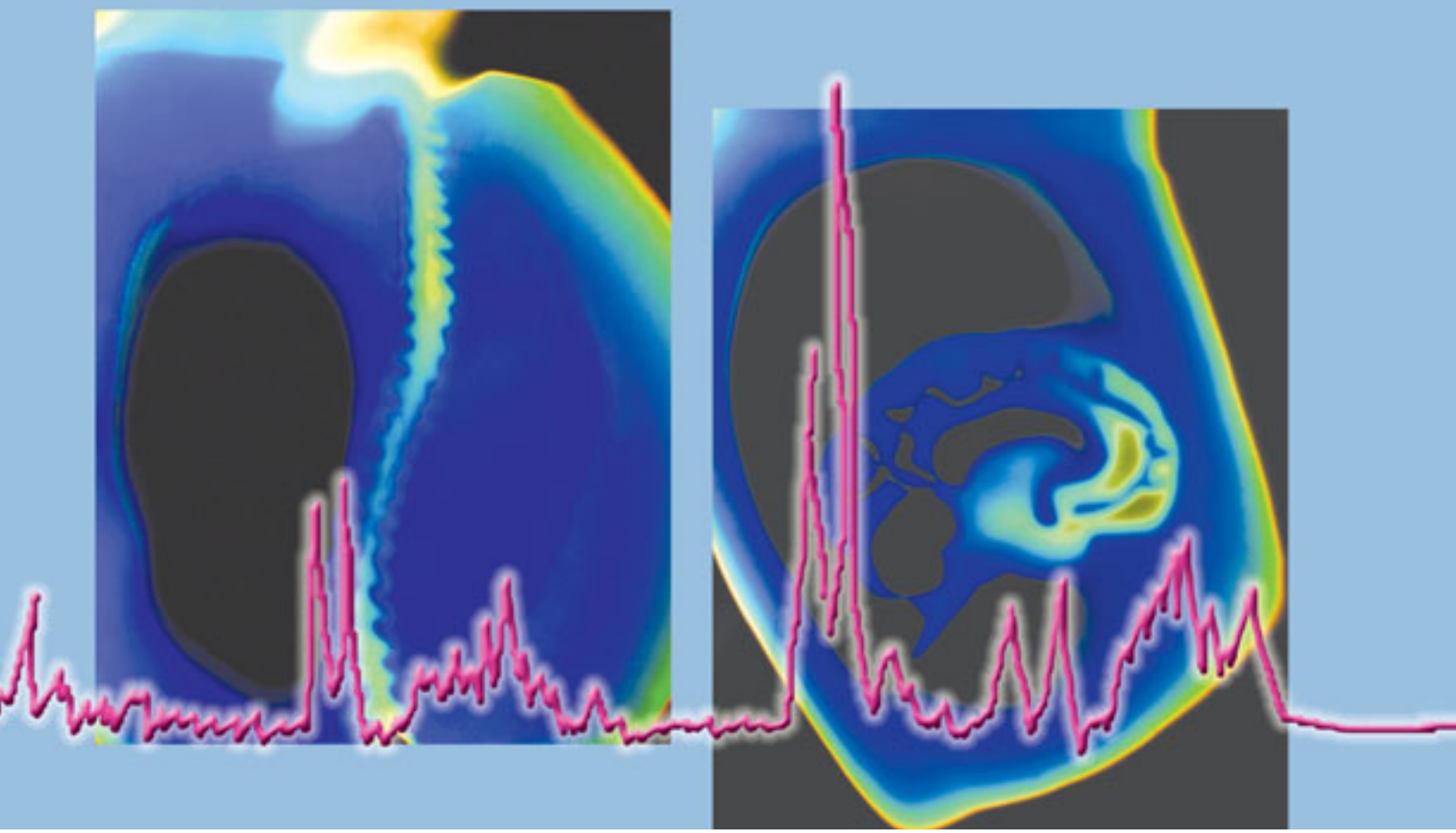


Clinical Proteomics:

What if your doctor could screen you for every known disease simply by taking a few drops of your blood? Or prescribe the best medication for your condition based on your personal protein fingerprint?

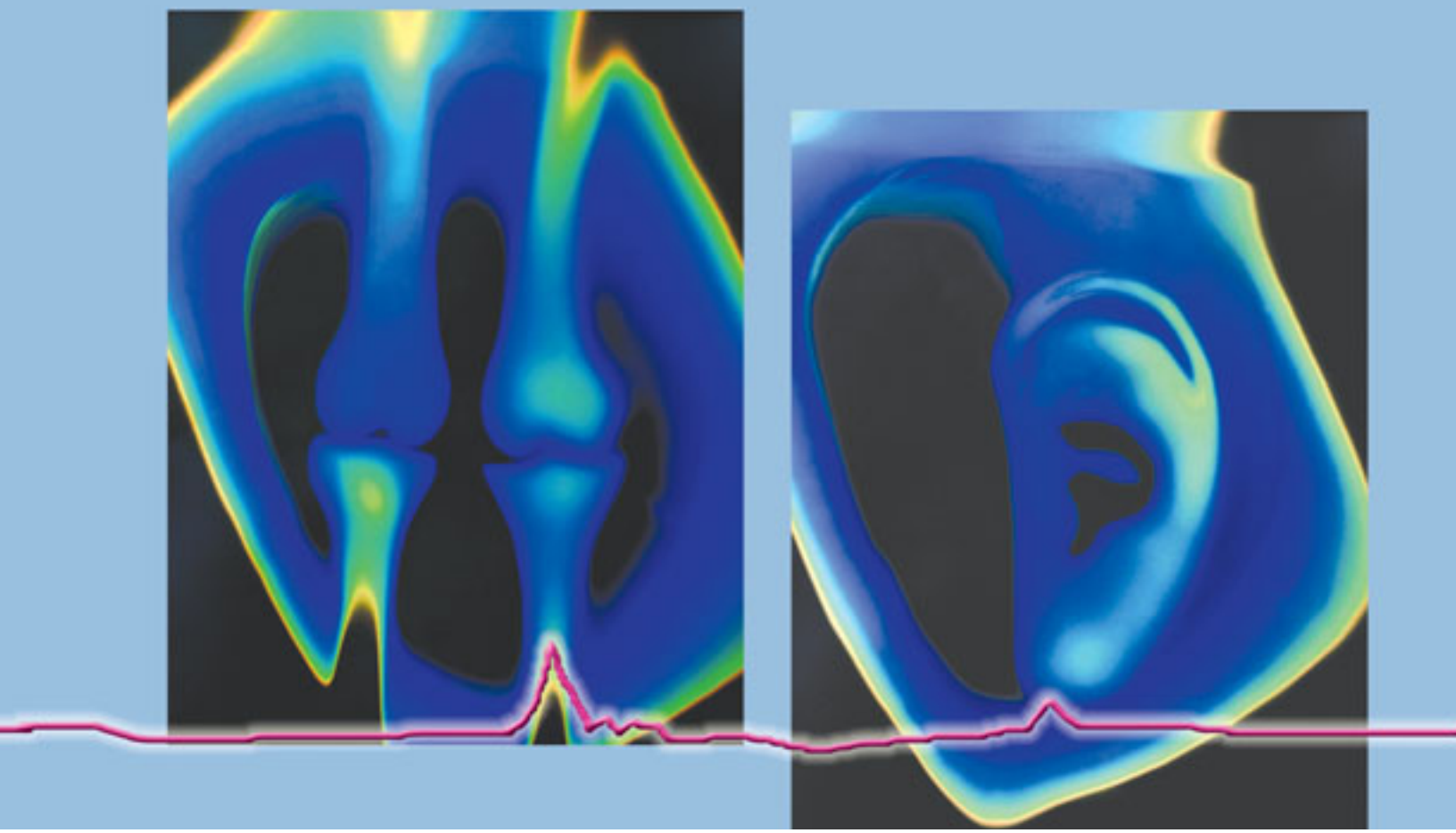
Such are the dreams of clinical proteomics researchers, who believe these possibilities can be realized just a few years from now. Although mass spectrometers are currently used in some places to screen newborns for metabolism defects, screening patients for all types of diseases would dramatically extend the reach of the mass spectrometer in the clinical laboratory. Clinical tests are expected to commence this fall to evaluate the technology for diagnosing ovarian cancer, and many researchers are studying several additional diseases for telltale MS peaks.

But is the technology really ready for the clinic? Some experts suggest that the technology has a long way to go before it can be implemented, and many doubt that the popular strategy of coupling surface-enhanced laser desorption/ionization (SELDI) protein chips with MS is the best approach.

Emanuel Petricoin of the U.S. Food and Drug Administration (FDA) acknowledges the controversy. "When we . . . first published on this, it generated a whole firestorm of criticism and excitement," he recalls. "It really comes down to people's feelings about mass spec as an instrument, as a clinical diagnostic, [and] the concept of patterns being diagnostics without knowing the underlying identity, which is foreign to people."

Petricoin says that time is critical for those who are likely to develop cancer. Clinical proteomics has the potential, he says, to prevent unnecessary biopsies and removal of organs. Many women at high risk for ovarian cancer, such as those with a family history of the disease, are having their ovaries removed without any biomarker indicating that cancer is present. "It's horri-

Clinical proteomics is **stirring up controversy,** causing some **to**



Are We There Yet?

Katie Cottingham

ble when you think about it, but it's a situation where if I was in their shoes, I might do the same thing," he says.

Although critics acknowledge the urgent need for better biomarkers, they caution that the method must be reproducible and specific, especially if the proteins used as biomarkers are not identified. Eleftherios Diamandis of Mount Sinai Hospital and the University of Toronto (both in Canada) agrees that proteomics could be a powerful tool for diagnosis, but he says the technique "needs a lot of work" before it can be applied to real clinical cases.

The method

The method that has become popular for experimentally screening patient samples is SELDI/MS. SELDI is similar to MALDI, except the surfaces of SELDI protein chips contain arrays of chromatographic surfaces with different properties, such as hydrophobic, cation exchange, anion exchange, and metal affinity. Thus, a SELDI chip retains molecules with certain properties, which are laser desorbed and ionized for analysis by MS. Ciphergen is the sole manufacturer of SELDI chips, which are made to fit onto Ciphergen TOF mass spectrometers and onto Applied Biosystems' QSTAR quadrupole TOF (QTOF) hybrid mass spectrometer via a special adaptor. Samples are run from patients with a disease (typically cancer) and from healthy patients, and bioinformatics software is used to compare spectra and determine the discriminating patterns of peaks (Figure 1).

Traditionally, researchers have used MS to identify new biomarkers and ELISA to detect them. Tim Veenstra of the National Cancer Institute says that in the case of clinical proteomics, however, "Mass spectrometry may not only be the tool that ultimately decides or identifies what the biomarker is, but it may also be the detection tool that does the assay for the biomarker."

wonder if it is premature to apply the science to real-world problems.

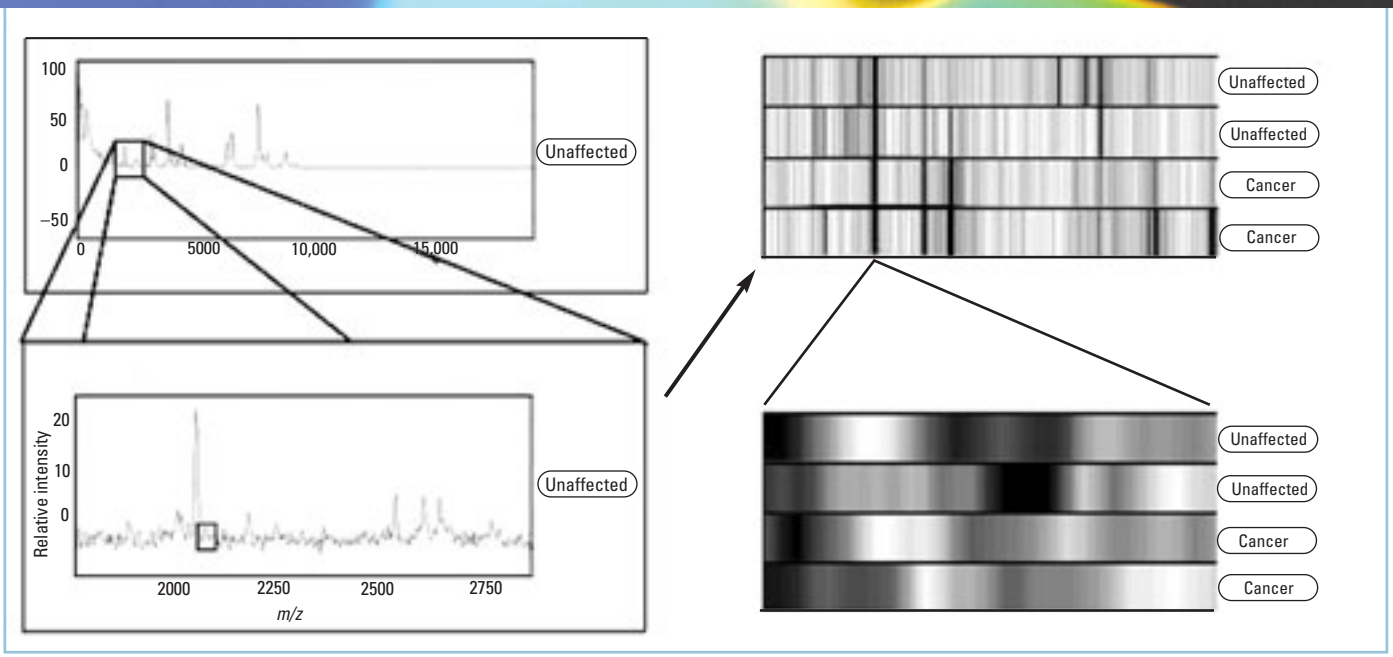


FIGURE 1. From mass spectra to fingerprints.

SELDI controversy

Most researchers agree that MS is a powerful technique with a bright future in clinical diagnostics, but what they can't seem to agree on is whether SELDI is the right separation method. Serum contains hundreds of thousands of molecules, but loading serum straight onto a mass spectrometer results in only a few peaks because six or seven highly abundant proteins will drown out the signals from other molecules. Thus, it is important to remove some of the abundant proteins prior to MS to be able to detect proteins expressed at low levels.

But SELDI limits what you can see. "You'll only see the tip of the iceberg," says Gyorgy Marko-Varga of AstraZeneca (Switzerland). He explains that a rapidly developing disease like cancer might produce enough factors to be seen by SELDI, but in slower progressing diseases or applications that analyze small amounts of tissue containing multiple cell types, the techniques used today may not provide the appropriate level of sensitivity. The resolution and abundance levels reached are crucial for novel biomarker findings. "In our experience, you need to move in and use larger volumes and . . . not that small chip surface, but you need a surface that is 1 million times larger [in order] to enrich those components that are in small amounts," says Marko-Varga.

Diamandis also says that SELDI surfaces preferentially bind high-abundance proteins, but he disagrees that tumors can shed proteins at levels high enough to be discerned by SELDI. He says, "There is no way that [the proteins Petricoin sees] come from the cancer cells by calculation." According to Diamandis, a small tumor produces minute amounts of biomarkers, which are diluted once they are released into the circulation. Diamandis estimates that a tumor would have to weigh 5 kg to produce enough proteins to be seen after serum is treated with SELDI. He speculates that the high-abundance proteins in the patterns are really coming from another organ, such as the liver, and he wonders how that can be a diagnostic for an ovarian tumor.

Most critics of the SELDI method propose using LC/MS as the diagnostic tool of choice. Many proponents of LC/MS say that SELDI is just not robust enough, although the advantages of MS cannot be denied. "With the [MS] technique, you generate solid data that provides convincing [evidence] that it is the right fragmentation because the accuracy of the measurement is extremely high using optimized protocols," says Marko-Varga.

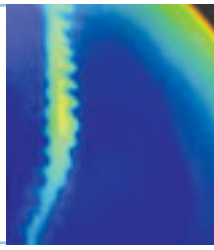
Although N. Leigh Anderson of the Plasma Proteome Institute thinks that LC/MS will eventually be more powerful than SELDI/MS, he believes that MS is not robust enough right now for use in a routine clinical test setting. He says that the ELISAs currently used in clinical laboratories are much more sensitive and accurate than what is available now for proteomics and biomarker discovery. But Anderson also says that using multiple markers doesn't limit researchers to just one technique. "I think just about anything is fair as a discovery technology as long as in the end you identify the proteins you're talking about, because then anybody could use any other technology to measure them."

Anderson, Diamandis, and Marko-Varga all cite reproducibility as a major problem of the SELDI/MS method, particularly if the identities of the peaks are unknown. "If you define an assay as a series of peaks in some kind of spectrum and you're going to say that gives the clinical result, it comes at a price—the price is you must be able to reproduce exactly the same set of peaks in anybody's lab, on anybody's machine, all the time," says Anderson. "The snag is that other people in other labs can't easily reproduce it. . . . This is a major problem." Irreproducibility between laboratories is exemplified in three prostate cancer studies, one of which was published by Petricoin's group, Diamandis points out. All three papers report different key discriminatory peaks.

The case for SELDI/MS

New data from Petricoin's team indicate that SELDI is retaining both high- and low-abundance proteins. But how can this be? In a paper scheduled for publication this year in the journal *Disease Markers*, Petricoin has sequenced some of the proteins comprising the patterns. "What we have found is that the fragments we've identified are actually tremendously amplified and

"If **the diagnostic** is proven over thousands of **samples** to have high specificity and **sensitivity**, then I **don't think** knowledge of the **proteins is critical to** the patient or his or her **physician**," says Veenstra.



bound to carrier proteins that can act almost as a molecular mop and sequester these biomarker fragments, these low-molecular-weight entities that are themselves the diagnostic," he explains. The carrier proteins, such as albumin, are likely sticking to the SELDI surface, bringing the less abundant biomarkers along with them.

Petricoin also argues that biomarkers needn't be in short supply within the serum. The tumor secretes enzymes that set off cascades of reactions with far-reaching effects, including proteolysis. "The first steps may be four or five molecules being released by a few cells, but the end results are enzymatic amplification cascades that set off a very specific chain reaction, the end product of which could be a million molecules clipped in the serum, and what you're looking at are those million clipped molecules by mass spec," he says.

Veenstra and Petricoin, who are collaborators, are skeptical of alternative methods, such as LC and ELISA. "Can you really do high-throughput diagnostics by LC/MS?" Veenstra asks. "The reproducibility and robustness of any capillary column are kind of a stretch. If we get a good capillary column packed and we run it for two to three months, we're pretty pleased with that," he says. It's too difficult to obtain consistent results with hundreds of capillary columns, according to Veenstra, whereas SELDI/MS is an easily automatable high-throughput method, something that is essential for a clinical test.

"While a mass spec as an instrument has limitations of sensitivity compared to an ELISA," says Petricoin, "the fact that the carrier proteins are amplifying the signal dramatically brings all these biomarkers to the range where you can detect them." But he and Veenstra think that the question of whether ELISA is a better technique is moot. "What we're seeing in serum may be primarily made up of fragments of larger proteins," says Veenstra. Consequently, it will be nearly impossible to generate antibodies to these biomarkers that only recognize fragments and not the intact form of a protein.

Interestingly, these researchers are also investigating alternatives to SELDI chips, though they are sticking to laser desorption and ionization. "What we're trying to do—I think this looks very promising—is we're trying to find a way that we can circumvent the [SELDI] protein chips and make this a straight MALDI TOF method," says Veenstra. The new method would be more flexible and cheaper than using SELDI chips.

As for accusations that SELDI/MS is not reproducible, Petricoin says there are two separate issues: variability among labs and the choice of mass spectrometer to use with the SELDI chips. He attributes differences reported in the three prostate cancer papers to the simple lack of a standard protocol among the laboratories. For instance, the groups did not use the same type of SELDI chips, which is one reason that different peaks were observed. He says that all of the proteins detected may be valid biomarkers. Differences in the ways the chips are prepared, the serum is prepared, and the samples are applied to the chip can vary the resulting pattern as well.

Within Petricoin's own laboratory, however, a standard operating procedure is followed, and it is now "very reproducible". But Petricoin admits that the original configuration—a SELDI chip mounted onto a low-resolution Ciphergen TOF mass spectrometer—was not always yielding the same results after a couple of months. "The problem was that the pattern was the same, but it was slightly shifted," he says. The Ciphergen system is a good instrument for discovery, says Petricoin, but for a clinical test, they needed more reproducibility. Veenstra now uses a high-resolution QTOF with which the group has obtained its most recent results. They will also run clinical trial samples on the QTOF instrument. Petricoin reports that results from the SELDI/QTOF system are much more reproducible over long periods of time.

Must biomarkers be identified?

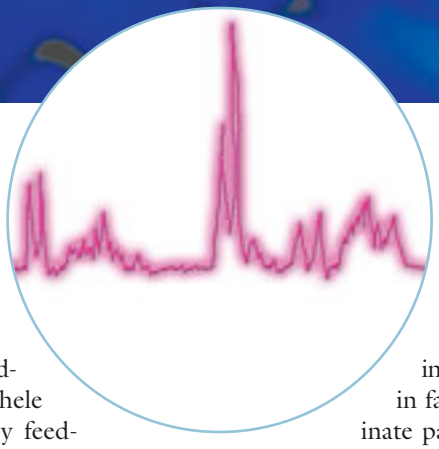
Another point of contention is whether biomarkers should be identified or if differing patterns of unknown MS peaks are sufficient for use as diagnostic tools. "A research scientist will argue it is critical to know what these are because they may shed some light on tumor progression and how the tumor acts in the body—that's true," Veenstra says. But the doctor–patient perspective is an important consideration, he points out, and the identities of each biomarker may not be important to either party. He says, "If the diagnostic is proven over thousands of samples to have high specificity and sensitivity, then I don't think knowledge of the proteins is critical to the patient or his or her physician."

Diamandis argues that at least some of the proteins should be identified to see if they make biological sense. Although past papers have not included the identities of the proteins making up the patterns, Petricoin and Veenstra say that they have now started identifying discriminatory proteins. But they also say that there's a precedent for using unknown proteins for disease diagnosis. For example, CA125 has been used for years to detect ovarian cancer and is approved by FDA, but researchers have only recently determined what the protein does. Petricoin also says that knowing a biomarker's identity as a protease or kinase does not necessarily add anything to its usefulness as a diagnostic.

FDA approval

There is a great need for additional biomarkers to diagnose disease, according to researchers, who report that FDA has approved very few new tests for biomarkers in the last 10 years. In light of how long it has taken other diagnostics to come to market, experts say it could take 10–20 years for new tests to make it through the discovery process to FDA approval.

A unique hurdle exists for proteomics-based tests—FDA does not have a formal regulation on how to submit such tests, though it has recently taken the first steps toward defining a policy. In



April 2003, FDA issued a draft guidance, called "Multiplex Tests for Heritable DNA Markers, Mutations, and Expression Patterns", which mainly focuses on DNA tests, including DNA microarrays. Although this guidance could also cover proteomics tests, Michele Schoonmaker of FDA says that preliminary feedback suggests separating the two types of tests.

"It's definitely a new avenue for us," says Schoonmaker. "We generally wait until we've had submissions before developing a guidance, but this has been more of a proactive stance based on discussions we've had with professional societies and industry." A final guidance will be issued once FDA officials have digested all the submitted comments.

Although the European Agency for the Evaluation of Medicinal Products (EMEA) is not charged with evaluating diagnostic tests, the agency does monitor developments in the field because of its impact on pharmaceuticals. Officials at EMEA say that it is still too early for detailed guidance on the issue. No specific regulation exists in Europe for proteomics tests, but current European Union directives would not exclude such tests, according to Marisa Papaluca Amati of EMEA.

Although Schoonmaker and Petricoin insist that FDA guidances do not require that particular tests be done in support of a new diagnostic or a new drug application, Marko-Varga interprets this as a sign of the times. "It's a clear message to the pharmaceutical industry that you'd better check out [the guidance] because this is something they will really demand from future applications to get FDA agreement." Schoonmaker says that FDA continually works with the scientific community to determine the most appropriate studies to validate technology.

Are we really there yet?

Clearly, the firestorm is still raging, and there is no consensus about whether SELDI/MS proteomics tests are ready for the clinical laboratory. Petricoin and his colleagues are conducting clinical trials this fall, and two of the largest private clinical laboratories, Quest and LabCorp, together with Correlogic Systems, Inc., are running their own trials for FDA approval. "The fact that these two giants are embracing and at least fairly evaluating this technology at the beginning of the road says something about how forward-thinking some people are," says Petricoin. But others warn that several obstacles must be overcome before the diagnostic and medical communities embrace the technology.

A standard procedure must be followed to ensure reproducibility, and the tests must be validated, which is something Anderson says is often neglected. "To get something into general use, you then have to go through and show that it's not a marker for any other disease. That's something that people in the research side generally aren't funded to do and aren't terribly interested in doing," he says. Petricoin says that in fact his group and others are able to discriminate patterns as indicative for only one disease by comparing them to patterns obtained from patients with other diseases.

Specificity and false positives are also at issue. Recent papers claim a specificity of 95 or 96%, which Anderson says leads to too many false-positive results for rare diseases. A high false-positive rate could send many people to surgery for unnecessary biopsies. He says, "The medical establishment is not immediately embracing these tests without knowing that they are really specific. They would like a test that is 99.99% correct. Until we get to that point, people are going to be a little bit lukewarm."

Petricoin believes that the specificity is already sufficient for screening patients at very high risk for developing cancer, though he admits it may not be quite ready for general population screening of rare diseases. When screening high-risk groups, he says, one doesn't attempt to discriminate a healthy person from someone who has cancer, but the goal is instead to discriminate a benign disease from cancer. "You already suspect something, and that's a different paradigm," says Petricoin. He also points to new data, published in July in *Expert Reviews in Molecular Diagnostics*. In this paper, his group reports 100% specificity and sensitivity for an ovarian cancer blinded study.

Many laboratories will have to buy mass spectrometers to handle the increased number of samples brought on by screening for many different types of diseases. The QTOF mass spectrometer Veenstra uses costs ~$300,000, a substantial investment for small hospitals. Although hybrid mass spectrometers have been used since the late 1990s in some states for newborn screening, experts say those particular instruments do not have MALDI capability; thus, even laboratories that routinely test newborns are not equipped for a SELDI- or MALDI-based diagnostic. But, Veenstra says, "I think the big expense will be your capital equipment up front, but beyond that, it's pretty comparable to other methods. I don't think the cost is prohibitive."

Many issues remain for scientists on the research and diagnostic testing sides. "There needs to be a level of cooperation and exchange between clinical proteomics and the existing diagnostic industry for this to work out," says Anderson. And *if* it works out, the rewards could be tremendous.

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Katie Cottingham is an associate editor of Analytical Chemistry.