BACKGROUND: Given the mounting evidence in favor of early pharmacologic and catheter-based interventions for patients across the spectrum of acute coronary syndromes, discovering novel diagnostically sensitive and specific biomarkers that provide biochemical proof of early or reversible myocardial injury could have a substantial positive impact on patient care.

CONTENT: To address unmet needs in disease biomarkers, investigators have turned to proteomics approaches. We describe advances in proteomics discovery technologies based on liquid chromatography–tandem mass spectrometry that facilitate the unbiased analysis of low-abundance blood proteins. We detail the development of emerging techniques to enhance the biomarker verification process, such as accurate inclusion mass screening, stable isotope dilution–multiple reaction monitoring–mass spectrometry (SID-MRM-MS), and stable isotope standards with capture by antipeptide antibodies, which combines the advantages of specific immunoaffinity enrichment of a target peptide with the structural specificity and quantitative capabilities of SID-MRM-MS. We highlight new assays incorporating these techniques for troponin I, a representative low-abundance cardiac biomarker, and interleukin-33, an emerging novel marker of myocardial stress for which no existing ELISA exists. We demonstrate that troponin I and interleukin-33 peptides have a linear, dynamic range spanning 4 orders of magnitude and limits of detection of approximately 0.5 μg/L back-calculated to the protein concentration.

CONCLUSIONS: There remain important unmet diagnostic and prognostic needs in cardiology. Advances in technology may allow proteomics to play a vital role in the discovery and validation of novel biomarkers to help fill those needs.

Circulating biomarkers of necrosis are the most commonly used cardiovascular biomarkers. By the late 1990s, a growing body of clinical data led to the establishment of the cardiac troponins, myocardial-specific structural proteins, as the cornerstone for the diagnosis of myocardial infarction (MI)\(^5\) (1). Troponin, however, offers far more than just improved diagnostic sensitivity and specificity. Several groups have demonstrated a powerful relationship between the increase in troponin and the risk of mortality in patients presenting with a non–ST-elevation acute coronary syndrome (NSTEMI), i.e., without classical changes on the electrocardiogram consistent with an acute injury pattern (2). New high-sensitivity assays have been developed that have lower limits of detection below the 99th percentile in a healthy reference population. With such assays, analysis of a single sample obtained at presentation from an individual with chest pain is approximately 90% diagnostically sensitive and specific for MI (3). Moreover, ultrasensitive assays are also being developed that have limits of detection (LODs) below the concentrations seen in a healthy reference population, allowing virtually all individuals to have a quantifiable troponin result (4). Such assays may make unstable angina (i.e., cardiac chest pain without biochemical evidence of myocardial injury) an obsolete diagnosis, although the clinical implications of very low-concentration values reported from ultrasensitive assays will need to be defined. These advances in troponin assays have set a higher bar for the discovery of new markers to diagnose myocardial ischemia.

With the growing appreciation for the multiple pathways contributing to atherothrombosis and its

---

\(^5\) Nonstandard abbreviations: MI, myocardial infarction; NSTEMI, non–ST-elevation acute coronary syndrome; LOQ, limit of quantification; CRP, C-reactive protein; BNP, B-type natriuretic peptide; LC-MS/MS, liquid chromatography–tandem mass spectrometry; SISCAPA, stable isotope dilution–MRM-MS; SID-MRM-MS, stable isotope dilution–MRM-MS; SISCAPA, stable isotope standards with capture by antipeptide antibodies; TnI, troponin I; IL, interleukin.
complications, additional biomarkers have garnered investigative support. With the advent of high-sensitivity assays for the inflammatory marker C-reactive protein (CRP), a more detailed look at supposedly normal CRP concentrations was possible. In doing so, investigators discovered that among apparently healthy individuals, higher concentrations of CRP that were still within the so-called normal range were associated with an increased risk of MI (5, 6). Whether CRP is directly causal remains contested (7), but the association with clinical events remains clear. Multiple other inflammatory biomarkers are under study, including lipoprotein-associated phospholipase A₂ (8), secretory type II phospholipase A₂ (9), oxidized LDL (10), myeloperoxidase (11), and growth differentiation factor 15 (12).

B-type natriuretic peptide (BNP) is a vasoactive hormone synthesized and secreted by the heart in response to increased left ventricular wall tension (13). The development of immunoassays to BNP has permitted rapid, noninvasive biochemical determination of left ventricular hemodynamic stress. The diagnostic and prognostic utility of BNP measurements was first demonstrated in the setting of heart failure, (14), a setting in which natriuretic peptides are now frequently measured. Increased BNP concentrations are an independent predictor of mortality in patients presenting with ACS (15).

We and others have also demonstrated the value of a multimarker approach to risk stratification in which different pathobiological axes are assessed biochemically (16, 17). Given that troponin is a marker of myocyte necrosis, CRP is a marker of inflammation, and BNP is a marker of left ventricular overload, we hypothesized that simultaneous assessment of all 3 markers—each representing a distinct biological axis—would offer complementary prognostic information and enable clinicians to more effectively risk stratify patients with ACS. When patients presenting with ACS were categorized based on the number of increased biomarkers at presentation, there was a near doubling of the mortality risk for each additional biomarker that was increased, a finding that was subsequently validated in an independent cohort (16). As described below, emerging proteomics techniques will allow us to “overlay” new biomarkers on existing multimarker scores. It is anticipated that some new markers will be uncorrelated or “orthogonal” to existing markers, thus providing additional information for cardiovascular disease management (18).

**Biomarkers to Guide Therapy**

In addition to providing powerful prognostic information, the determination of biomarkers such as troponin helps clinicians tailor therapeutic interventions. In NSTEACS patients with increased troponin concentrations, treatment with the glycoprotein IIb/IIIa inhibitor abciximab was associated with a substantial reduction in death or MI, whereas no benefit was seen in patients who did not have increased troponin (19).

Similarly, we have shown that troponin increases could be used to identify patients in whom a strategy of early cardiac catheterization was of particular benefit (40% risk reduction in death, MI, or rehospitalization for ACS vs 29% risk increase in patients without increased troponin) (20). To that end, the most recent College of Cardiology/American Heart Association guidelines for the management of patients with NSTEACS now recommend routine measurement of troponin to guide therapy (21).

**Unmet Needs with Current Cardiovascular Biomarkers for ACS**

Although troponin is an excellent marker of MI, marked increases are not apparent until several hours after the onset of ACS. Given the mounting evidence in favor of early pharmacologic and catheter-based interventions for patients across the spectrum of ACS (22, 23), discovering novel, diagnostically sensitive, and specific biomarkers that provide biochemical proof of early myocardial injury could have a substantial positive impact on patient care.

Furthermore, although we have several markers of irreversible myocardial necrosis, a major current deficiency is that we have no satisfactory markers of reversible myocardial ischemia (24). The development of such markers would permit biochemical confirmation of unstable angina, which must currently be diagnosed by a combination of a history consistent with typical angina pectoris and electrocardiographic findings of labile ST-segment and T-wave changes. This approach is often unsatisfactory, however, because of the transient nature of electrocardiographic changes and the subjective nature of history taking, particularly in the ever-increasing subsets of elderly and diabetic patients. Faced with these limitations, physicians will typically order a stress test to help confirm or exclude the diagnosis of myocardial ischemia. However, this approach also has its limitations. A standard exercise stress test has a diagnostic sensitivity of only 60% (and <50% for single-vessel disease) and a diagnostic specificity of only 70% (25). The addition of myocardial perfusion imaging with agents such as 201thallium or 99mTc-sestamibi improves the operating characteristics of the test, but adds more than $2500 to the cost. In addition to myocardial ischemia, other pathophysiological pathways are in need of reliable biochemical detection,
including endothelial cell dysfunction, oxidative stress, and platelet aggregation.

Identification of biomarkers linked directly to the underlying pathophysiology of ACS will enable more precise diagnosis and prognosis determination. For example, plaque rupture is the proximal event precipitating an ACS. To that end, measurement of circulating endothelial cells and pregnancy-associated plasma protein A, a matrix metalloproteinase expressed in atherosclerotic lesions, appears to add to existing diagnostic strategies in pilot studies (26).

Proteomics Technologies for Candidate Biomarker Discovery

Despite the enormous potential of biomarkers to revolutionize clinical practice and improve patient care through the development of new molecular–based diagnostic and prognostic tests, in the last 5 years very few new protein markers for measurement in plasma or serum have been cleared by the US Food and Drug Administration (27). The reasons for this serious discrepancy have been explored in several recent reports and reflect the long and difficult path that a biomarker must take from initial candidate discovery to clinical use (28). The problems derive from the high false-discovery rate of -omics methods (regardless of technology used), and more specifically to proteomics, the huge dynamic range of human plasma, and a lack of robust methods for biomarker verification in large clinical sample sets.

To address these issues, considerable advances have been made in proteomic discovery methods over the past 5 years, owing to improvements in analysis methods as well as advances in instrument sensitivity. The advent of new chromatographic techniques to deplete plasma samples of high-abundance constituents has also been of critical importance. It is now common for differential analysis of tissue or plasma by multidimensional liquid chromatography–tandem mass spectrometry (LC-MS/MS), the workhorse tool for unbiased discovery, to provide confident identification of thousands of proteins (29, 30). A schematic of a discovery approach, as well as the verification approaches detailed below, is outlined in Fig. 1. To access proteins at lower abundance, however (e.g., less than 100 μg/L in plasma, concentrations at which many known protein biomarkers such as carcinoembryonic antigen, prostate-specific antigen, and the troponins occur), analyses must employ multidimensional fractionation at the protein and/or peptide level. Thus, a single patient sample may need to be expanded into aliquots of up to a 100 subfractions, each requiring lengthy LC-MS/MS analysis. It is not uncommon for the analysis of a single pre/postintervention or case/control sample pair to take up to 2 weeks of on-instrument time. The amount of time required limits the numbers of samples that can be practically analyzed to typically a dozen (or fewer) case vs control or pre- vs postintervention comparisons. In the face of these obstacles, a growing consensus is that discovery work should be focused on disease models for which small cohorts of well-phenotyped samples might be particularly informative. Regardless, sample numbers are very small relative to the high dimensionality of the proteome (100 000 or more possible components when posttranslational modifications and other variants are taken into account) and the scale of normal variation in the human population. Thus, a substantial fraction of proteins “discovered” in these experiments are false positives arising from biological or technical variability. Clearly, discovery experiments do not lead to biomarkers of immediate clinical utility, but rather produce candidates that must be qualified and verified (28).

Proteomics Technologies for Candidate Biomarker Validation

Until recently, verification technologies have not been available that are capable of testing large numbers of protein biomarker candidates emerging from discovery -omics experiments in large (>1000) sample sets. In principal, antibody-based measurements could be used at all steps in the validation process. However, the required immunoassay-grade antibody pairs exist for only a small number of the potential candidate biomarker proteins. Indeed, in many discovery efforts to date, not even 1 antibody exists for many of the identified proteins. Developing a new, clinically deployable immunoassay is expensive and time consuming, characteristics that restrict development to a short list of already highly credentialed candidates (28). Clearly, for the many new candidate biomarkers, non–antibody-based verification technologies are required to complement antibody-based techniques.

Ideally, new techniques must have reasonable assay-development timelines and low assay-development cost, be effectively multiplexed to analyze for 10–50 proteins in a single analysis, have low patient sample consumption (approximately 100–500 μL or less for the 10–50 proteins), and achieve a throughput of 100s of patient plasma samples with good assay precision. We refer to this activity as verification, and its goal is to identify those few candidate protein biomarkers from the initial list of potentially hundreds that are worth advancing to traditional candidate validation studies using assays deployable on a clinically approved analysis platforms (31, 32).

To achieve the goals stated above, investigators have been actively engaged in several key advances in
MS-based verification technologies. The first involves the use of accurate inclusion mass screening (AIMS), made possible by the exquisite mass accuracy of the most recently developed mass spectrometers. This technique was developed as an initial bridge between discovery-based proteomics and quantitative assay configuration that uses antibodies or other approaches such as multiple reaction monitoring mass spectrometry (MRM-MS) (see below). In AIMS, an inclusion list is populated with the accurate masses of signature peptides derived from the high-priority candidate proteins from discovery experiments (33). Masses on the inclusion list are monitored in each scan on the MS system and MS/MS spectra are acquired only when a peptide from the list is detected with both the correct accurate mass and charge state. AIMS enables rapid, semiquantitative qualification of approximately 100 proteins/week in patient blood (33). It is well suited as a bridge between discovery and targeted, quantitative MS-based assay development. AIMS enables investigators
to triage (qualify or discard) a large number of biomarker candidates based on highly specific and sensitive detection of peptides in plasma prior to committing to subsequent time and resource intensive steps. It is important to note that the AIMS method is equally useful for proteins containing modifications such as phosphorylation or sequence isofoms provided that peptides containing these modifications are released upon digestion of the proteins with trypsin (or other enzymes) and are compatible with LC-MS analysis.

The core technology that is beginning to emerge for verification by quantitatively assaying candidate biomarkers in blood is stable isotope dilution–MRM-MS (SID-MRM-MS). This method has been very successfully used for quantification of small molecules (e.g., hormones and drugs and their metabolites) in pharmaceutical research and more recently in clinical laboratories, but has only recently begun to be seriously applied to measure proteins. Use of SID-MRM-MS for protein assays is predicated on measurement of signature or proteotypic tryptic peptides that uniquely and stoichiometrically identify the protein candidates of interest. MRM-based assay development starts with selection of 3–5 peptides per protein (34). Synthetic, stable isotope-labeled versions of each peptide are used as internal standards. These exogenous heavy-labeled peptide standards are identical to their endogenous, analyte peptide counterparts with the sole exception that they weigh more (typically 6–10 Da more, depending on the label used). Specific fragment-ion signals derived from the endogenous unlabeled species are measured and compared to those from the exogenous labeled peptides. The ratio of these values provides a precise measure of the concentration of the corresponding protein.

SID-MRM-MS assays have several distinguishing features relative to conventional immunoassays. First, the analyte detected in the MS can be characterized with near-absolute structural specificity—something never possible with antibodies used alone. Furthermore, MRM-MS [and the related approach of stable isotope standards with capture by antipeptide antibodies (SISCAPA), described below] can be used without modification to develop assays for modified peptides (e.g., phosphopeptides) and peptides spanning sites of slice variation or sequence mutation. This provides a potentially critical quality advantage, especially in cases in which immunoassays are subject to interferences. Second, MRM assays can be highly multiplexed such that hundreds of peptides derived from dozens of proteins can be measured during a single one-hour analysis, with excellent CVs. (34). Third, all of these measurements can be done on approximately 100 μL of plasma or less.

Many biomarkers of current clinical importance, such as prostate-specific antigen, carcinoembryonic antigen, and the troponins, are present in the mid–nanogram per liter to low–microgram per liter range in plasma. It has recently been demonstrated that a combination of abundant protein depletion combined with minimal fractionation of tryptic peptides by strong cation-exchange before SID-MRM-MS provides limit of quantification (LOQ) signal-to-noise ratios of >10 in the 1–20 μg/L range, with CVs of 10%–20% at the LOQs for proteins in plasma (34), including the configuration of assays for a number of known markers of cardiovascular disease (35), providing additional proof of the power of MRM methods for configuring assays for proteins for which antibody reagents are not available. Thus, MS is rapidly becoming an important technology to complement immunoassays for biomarker validation.

Although dozens of assays can readily be configured and run using this approach, one drawback is that there is still upfront sample processing required to achieve low-nanogram LOQs, which restricts sample throughput compared to immunoassays. Detection of proteins in the mid-to-low–nanogram per liter range by use of this approach is not currently possible because of current limits to MS detection, although improvements in instrument design on the near horizon may help break this barrier (36). For these reasons, investigators have been exploring SISCAPA, which combines the advantages of specific immunoaffinity enrichment of a target peptide with the structural specificity and quantitative capabilities of SID-MRM-MS (37). In this approach, antipeptide antibodies are made against the selected signature tryptic peptides from the proteins of interest. Following digestion of the plasma and addition of known amounts of stable isotope-labeled standard peptide, both added and sample-derived versions are specifically enriched and the relative amounts measured by MRM. Thus, the MS serves as the second antibody. Recent studies suggest that more than a thousandfold enrichment can be achieved for plasma digest peptides using this approach, (38), and that SISCAPA assays can achieve low–microgram per liter LOQs in plasma with CVs <20% (35). The coupling of SISCAPA to magnetic bead–handling robotics that automate peptide capture, wash, and elution steps can markedly improve throughput.

To evaluate the application of this approach from unprocessed plasma, we generated antipeptide rabbit polyclonal antibodies to 2 peptides derived from troponin I (TnI) and interleukin (IL)-33 (35). TnI was chosen as a representative low-abundance cardiac marker. IL-33 was chosen because it is the ligand for ST-2, an emerging biomarker of myocardial stress, and no ELISA currently exists for this protein. A 9-step pro-
tein response curve, in which recombinant TnI and IL-33 proteins were spiked into plasma from a healthy female donor is shown in Fig. 2A. Samples were reduced, alkylated, and trypsin digested before peptide capture on antibody-conjugated magnetic beads. LC-MRM-MS/MS was performed in triplicate on the eluate of the antibody capture. The response curves indicate that the TnI and IL-33 peptides have a linear, dynamic range spanning 4 orders of magnitude and LODs of approximately 0.5 μg/L back-calculated to the protein level. Then to demonstrate applicability to clinical samples, SISCAPA was used to analyze peripheral plasma sampled at baseline and at 10 min, 60 min, 2 h, 4 h, and 24 h after treatment from 5 patients who underwent a planned MI for hypertrophic cardiomyopathy (39). Samples were digested and subjected to antibody-capture of the target peptide derived from TnI before LC-MRM/MS analysis. Fig. 2B shows that the concentration of TnI increases with time after cardiac injury in 5 planned MI patients and is consistent with clinical results ($R^2 = 0.82$, not shown). Thus, LC-MS/MS–based methods hold tremendous promise for facilitating the transition from biomarker discovery to validation, although at present LOQs of the SISCAPA technique cannot match those of newer immunoassays for the cardiac troponins.

Development of a Proteomics Workflow

The aforementioned hurdles—and the emerging tools to overcome them—must be considered in the design of studies with the best likelihood of identifying useful blood biomarkers. One potential workflow is outlined in Fig. 1. With the limited throughput inherent to deep mining of the proteome for discovery, investigators may focus their initial investigative efforts on tissues and proximal fluids, which are enriched for potential signals of interest. Discovery studies employ multidimensional sample fractionation at the protein and/or peptide level followed by intensive MS-based analyses. Efforts can then turn to increasingly larger numbers of peripheral blood samples for follow-up studies. The sequential integration of targeted techniques such as AIMS, SIDS-MRM, and SISCAPA may serve to weed out from the discovery effort false positives arising from biological or technical variability. Subsequent to these efforts, ELISA reagents would be generated for the top candidates, to be further evaluated in large, het-
Clinical Application of Novel Biomarkers

Returning from the bench to the bedside, adoption of a novel biomarker into clinical practice will likely hinge on demonstration that measuring the biomarker’s concentration should trigger a clinical intervention, pharmacologic or otherwise. For prognostic biomarkers, patients at higher risk for adverse events will, by definition, be afforded greater absolute risk reduction for a given relative risk reduction from a therapy, and hence a smaller number of these patients will need to be treated to prevent an adverse event. But ideally, pathobiologically relevant prognostic biomarkers would identify a novel treatment interaction with a larger relative risk reduction and thus afford even greater absolute risk reduction.

Novel biomarkers that are true risk factors and faithfully serve as surrogates for clinical outcomes have the potential to markedly diminish the cost of drug development. For example, the ability to detect atherosclerotic plaque destabilization biochemically would allow researchers to screen and identify more easily pathobiologically relevant biomarkers that could then be tested in larger and more expensive phase III trials. For example, myeloperoxidase concentrations were used as a surrogate endpoint in the testing of a 5-lipoxygenase–activating protein inhibitor in MI (40). Moreover, pathobiologically relevant biomarkers may also serve as targets for drug development. Examples of drugs being developed based on compelling biomarker data include inhibitors of lipoprotein-associated phospholipase A2 and secretory phospholipase A2.

In conclusion, there remain important unmet diagnostic and prognostic needs in cardiology. Advances in technology may allow proteomics to play a vital role in the discovery of novel biomarkers to help fill these needs.

References

Proteomic Tool Integration for Biomarkers of Myocardial Injury


