

The Role of Proteomics in Clinical Cardiovascular Biomarker Discovery*

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Cardiovascular disease remains the most common cause of death in the developed world and is predicted by the World Health Organization to kill ~20 million people worldwide each year until at least 2015. In light of these figures, work on producing superior tools for clinical use in the cardiovascular field is intensive. As proteins are the primary effectors of cellular function, a significant majority of this work focuses on the role of proteins in the cardiovascular system in physiological and pathological states in order to outline both mechanisms and markers of disease. One of the most effective ways to investigate these on a global basis is through proteomic analysis, which allows for broad spectrum screening of cellular protein or peptide complements during cardiovascular pathogenesis. Furthermore, specific technologies are now available to screen animal model or human blood samples for novel, improved markers of chronic disease states, such as atherosclerosis or for earlier indicators of acute myocardial stress, including ischemia/reperfusion injury and heart failure. This review summarizes current literature on the key aspects of proteomics and peptidomics related to clinical cardiovascular science. *Molecular & Cellular Proteomics* 7:1824–1837, 2008.

The aim of cardiovascular proteomic studies is 2-fold: firstly, proteins which are directly and causally involved in functional or disease processes can be highlighted and, with further work, targeted by therapeutic intervention, and these can be called “mechanistic studies”; secondly, proteins which are altered in abundance or modified in a predictable manner in response to disease states can be located and used as markers for that same disease state (1). These studies are less focused on elucidating the mechanism of disease, instead aiming to improve clinical tools relating to disease-state and can thus be referred to as “biomarker studies”. This review will discuss the current state and potential future directions of each of these classes of cardiovascular proteomics investigation.

Mechanistic Proteomics—Mechanistic proteomic studies involve large-scale protein profiling of a proteome or subcel-

lular proteome, for example mitochondrial or nuclear proteins (2), with the goal of identifying alterations to protein abundance or post-translational modification that may be involved in pathogenesis, in either a causal or a consequential sense. Cardiovascular diseases (CVD)¹ have been subjected to extensive study in this way, covering all major pathological conditions, including what may be the two primary causes of CVD-related morbidity and mortality: ischemic heart disease (IHD) and heart failure (HF). IHD involves a variable degree of coronary stenosis leading to a myocardial oxygen supply-demand imbalance (ischemia), followed by reperfusion in a timeframe dependent on the ability of the clinician to detect and remove the underlying pathology, generally by interventions including thrombolysis and angioplasty. IHD is primarily thought of as an acute condition because of the sudden nature of clinical presentation, though long-term intermittent ischemia/reperfusion (I/R) injury can lead to chronic pathologies. In contrast, HF is the condition whereby the output of the heart is insufficient to meet the demands of the body and is usually preceded by one of several kinds of cardiomyopathy (hypertrophic, dilated, or restrictive). As such, HF is a result of chronic insult and is considered an “end-stage” pathological state. In essence, the underlying cardiac pathology causes a reduction in contractile capacity, forcing the heart to increase systolic pressure to maintain stroke volume and hence cardiac output. At some stage, this compensatory mechanism can no longer offset the reduction in function, leading to failure. The heart can fail on the right or left side, with the former reducing the ability of the heart to oxygenate sufficient blood, the latter its ability to distribute it to tissues. Both HF and IHD have been extensively profiled in the search for mechanisms of pathology.

Ischemic Heart Disease—IHD can lead to several distinct pathological outcomes depending on the length and severity

¹ The abbreviations used are: CVD, cardiovascular disease; ACS, acute coronary syndromes; BNP, B-type natriuretic peptide; CK-MB, creatine kinase muscle/brain isoform; cTn, cardiac troponin; HF, heart failure; H-FABP, heart-type fatty acid binding protein; I/R, ischemia/reperfusion; IHD, ischemic heart disease; IMA, ischemia-modified albumin; IPC, ischemic pre-conditioning; MLC, myosin regulatory light chain; MRM, multiple reaction monitoring; PCO, ischemic post-conditioning; ROS, reactive oxygen species; RPC, remote pre-conditioning; Tn, troponin; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; MS, mass spectrometry; LC, liquid chromatography; ELISA, enzyme-linked immunosorbent assay; SELDI, surface-enhanced laser desorption ionization; 2-DE, two-dimensional electrophoresis.

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of ischemia and consequent variation in myocyte damage. For instance, prolonged ischemia prior to reperfusion will lead to irreversible damage to the myocardium and a significant to severe loss of contractile capacity as myocytes undergo apoptosis and necrosis (infarction). If the initial ischemia is followed by incomplete reperfusion, *i.e.* to a level of perfusion that is low but above zero, a contractile abnormality known as myocardial hibernation will develop. Of somewhat more interest, in a mechanistic sense, is the myocardial injury sustained after brief periods of ischemia, after which limited or no necrosis takes place but a significant, eventually reversible, contractile deficit is still observed. This effect is known as myocardial stunning (3). At the physiological level, ischemia is characterized by a switch to anaerobic metabolism to maintain ATP production; however energy demand exceeds supply, and effective contraction becomes compromised. Furthermore, elevated lactic acid levels caused by anaerobiosis reduce the intracellular pH leading to reverse activity of the sodium/calcium exchanger and increased Ca^{2+} levels within myocytes. Upon timely reperfusion, mitochondrial oxidative phosphorylation resumes, ATP levels rise, and intracellular acidosis is resolved. Reperfusion, however, is immediately marked by a dramatic surge of reactive oxygen species (ROS). Scavenging of ROS by antioxidant intervention can provide significant protection against contractile dysfunction. The proteomic profile of tissue following reversible injury has been extensively examined in an attempt to elucidate specific proteins responsible for the phenotype of contractile loss (*e.g.* sarcomeric or cytoskeletal proteins) and the signaling networks underpinning the disease phenotype, *i.e.* protein kinase cascades (4–6). In the case of the former, there is almost no part of the molecular contractile apparatus, which has not been at some stage implicated in contributing to the observed loss of function. Our group has shown correlation between phosphorylation, deamidation, and proteolytic cleavage of myosin regulatory light chain-2 (MLC-2), an essential protein in contraction, and the functional derangement observed in myocardial stunning (Fig. 1; (7)). This correlation was subsequently extended to irreversibly injured tissue, prompting suggestion that MLC-2 may be a contributor to the post-ischemic myocardial phenotype (7). Because only 10% of the total pool of MLC-2 is degraded during injury; however, it is likely that this alteration is not the sole mediator of contractile dysfunction and provides further evidence that I/R injury is multifactorial. For example, others have also highlighted degradation of MLC-1 after both brief and extended regional ischemia (8, 9).

In addition to MLC variants, all three members of the troponin (Tn) complex have been implicated in I/R injury. Several groups have observed signs of troponin I (TnI) proteolysis in post-ischemic myocardium (10–12), and as part of the troponin regulatory complex that mediates excitation-contraction coupling at the level of the myofibrils; TnI degradation provides an attractive hypothesis to explain decreased myocar-

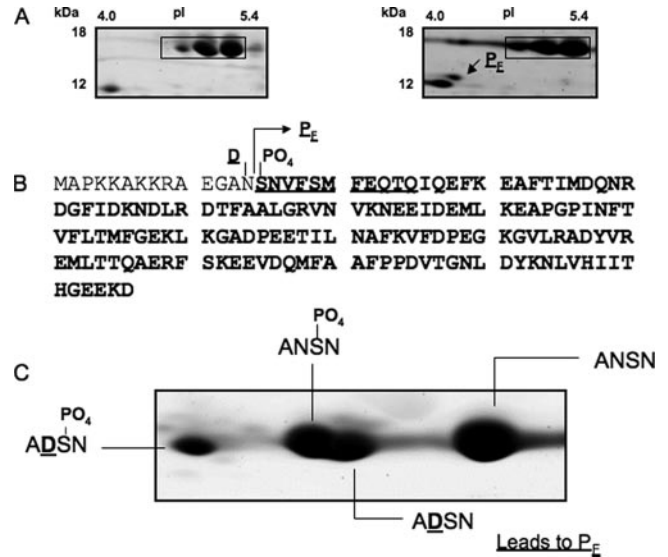


FIG. 1. **Degradation of MLC-2 during I/R injury.** A, 2-DE gels of myocardial proteins following baseline (control) perfusion (*left*) and 60 min ischemia/60 min reperfusion (60I/60R) (*right*). Boxed region shows spots corresponding to native MLC-2 mass; arrow indicates presence of MLC-2 cleavage product. B, MLC-2 amino acid sequence showing sites of post-translational modification. Asn-14 deamidated to Asp-14; phosphorylation of Ser-15 and site of cleavage to form P_E between residues 14 and 15. C, MLC-2 variants as detected by 2-DE and MS. Because P_E is not phosphorylated we conclude that it is derived from the deamidated (a signal for protein breakdown), non-phosphorylated pool of MLC-2.

dial contractility. TnI degradation, however, appears to be a model-dependent phenomenon, with other groups working on different species failing to observe similar results in the absence of necrosis (6, 13). Possible explanations include the suggestion that I/R-induced TnI proteolysis is limited to small rodents (14), or that degradation is a result of processes not related to I/R (15). Modifications to both troponin T and C (TnT and TnC) have also been implicated in I/R injury, with translocation of TnC from the sarcomere to the cytosol being observed after brief ischemia in rabbits (6) and TnT degradation noted in models of ischemic stress (16).

Stunning is effectively the result of two separate events (3); firstly, myocardial ischemia, and then the subsequent reperfusion that results in a massive influx of damaging ROS and Ca^{2+} -overload. Global proteomics studies of early, reversible I/R injury identified several contractile proteins that were significantly altered in animal models. Subsequent studies using the same models sectioned into brief ischemia with and without reperfusion and in the presence of free radical scavengers (17) clearly show that damage to contractile proteins occurs only following reperfusion and is not seen in myocardium “protected” by ROS scavengers. This provides substantial evidence that reversible contractile dysfunction is mediated by ROS at the physiological level and that limitation of “reperfusion injury” will significantly reduce protein damage and clinical symptoms. Such protection is now often afforded

clinically by ischemic pre-IPC or post-conditioning (PCO), where the heart can be “readied” for full reperfusion by very short bursts of I/R. IPC is a series of short, reversible coronary artery occlusions given prior to sustained ischemia (18), which has been shown to significantly reduce both contractile deficit and infarct size in short and prolonged I/R. However, the clinical utility is limited as patients most frequently present post-ischemic onset. IPC is evident in those with pre-infarction angina and mimicked with exercise training for those “at risk” (19), as well as to reduce injury associated with cardiopulmonary bypass surgery. PCO is performed following ischemia, but prior to full reperfusion (20), and as such is clinically relevant in reducing injury associated with acute myocardial infarction.

Clinical interventions by the administration of pharmacological agents have centered on ROS scavengers and the inhibition of Ca^{2+} -overload, yet much recent success has centered on agents that modify protein kinases and signaling pathways. These include fasudil (a Rho-kinase inhibitor) (21) and sildenafil (22), bradykinin, as well as experimental inhibitors of glycogen synthase kinase 3- β and p38 (23, 24). After it was realized that activated protein kinase cascades were able to salvage or protect myocardium subjected to I/R, proteomic studies have begun to reveal the associated signal pathways, in particular those involved in protection through IPC. In one of the relatively few proteomics investigations of IPC, pharmacological actions to mimic pre-conditioning stimuli (e.g. opening of mitochondrial ATP-sensitive potassium channels; mito- K_{ATP}) (25) were applied to rabbit ventricular myocytes and analyzed by multi-gradient 2-DE (26). This work revealed novel phosphorylation sites on MLC-1 (with implications for direct functional effects of IPC and I/R) as well as post-translational modification of several mitochondrial proteins involved in oxidative metabolism. Because mitochondrially derived excess ROS release is known to be one of the pathological hallmarks of I/R injury (3) as well as a mediator of pre-conditioning protection (27), determining modifications to these proteins could make a significant contribution to the understanding of IPC and PCO at the molecular level. A further variant of IPC, termed remote pre-conditioning (RPC), has also been analyzed using proteomic techniques. RPC involves ischemic insult to organs other than the heart prior to index myocardial ischemia and results in protection from subsequent myocardial I/R injury of a degree comparable with IPC. Lang *et al.* (28) analyzed serum by 2-DE in an attempt to detect a humoral triggering mechanism for RPC. As analysis of serum or plasma presents several challenges to proteomic studies (further discussed below), the fact that the investigators were not able to pinpoint a humoral candidate for RPC triggering is unsurprising, though it does not rule out its existence. As with all 2-DE-based proteomic studies, proteins, which lie outside the range of investigation, will not be detected. Therefore, the possibility remains that very hydrophobic, high, or low mass or very low abundance proteins may

carry the RPC signal from source to site. This study did, however, locate fragments of albumin seemingly released as a consequence of ischemia, which may support other data showing albumin modifications post-I/R, including changes in copper/cobalt binding capability (ischemia-modified albumin (IMA) (see below).

The physiological factors underpinning I/R injury have been well described, yet the precise signaling events responsible for mediating these are poorly understood. Even in the past year alone, a number of studies have shown a role for several signaling proteins in I/R, including PKC- β (29), AMP-activated protein kinase (30), phosphatidylinositol 3-kinase/Akt (31), p38 MAPK (32), Erk1/2 (33), and the inhibitory κ B kinase β -subunit (34). Each of these studies is undoubtedly useful, but an integrative analysis examining signaling on a global scale in myocardial I/R is needed. Furthermore, there is also debate surrounding the signal events that lead to cardioprotection afforded by IPC and PCO (35–37). Some signal pathways appear to be activated in both IPC and PCO, including adenosine receptors, phosphatidylinositol 3-kinase (PI3K)-Akt, Erk1/2, and eNOS (36, 38), whereas pre-conditioning-specific kinases (whether ischemic or pharmacological) include the ε - and δ -isoforms of protein kinase C, glycogen synthase kinase, and the MAPK family (36, 39–42). Elucidation of I/R signaling pathways is further complicated by “molecular hubs” such as PKC- ε , which have been shown to interact with large numbers of effector proteins (stress response, structural proteins, etc.), as well as other signal molecules, in response to pre-conditioning. It is clear that a global analysis of signaling during I/R injury and following both pharmacological and physiological intervention is necessary to correlate these conflicting data and provide novel insights into how signal pathways are integrated during myocardial pathogenesis.

Heart Failure—Proteomic studies of HF subsequent to cardiomyopathies have revealed changes to proteins in many different functional classes and have been reviewed extensively (43–45). 2-DE has been a technology of choice for some time to investigate protein alterations associated with cardiomyopathies and HF. In recent times it has been utilized to investigate myofilament-associated and cytosolic proteomes in failing mouse hearts and identified changes in abundance of, and/or modifications to, metabolic, structural, and contractile proteins (46). Studies in a canine model of I/R leading to HF also identified changes to several key classes of proteins, but in some particularly noteworthy cases the findings conflicted with those determined in rodents (8). For example, the increase in abundance of creatine kinase M chain observed in mice (46) was almost precisely reversed in the canine model (8). As is postulated to be the case for TnI proteolysis in acute I/R, species and/or model-dependent factors may determine the outcome. Stress response proteins have also been noted to correlate with pathology in HF, as is the case with IHD (47–48). Published data on studies of HF in

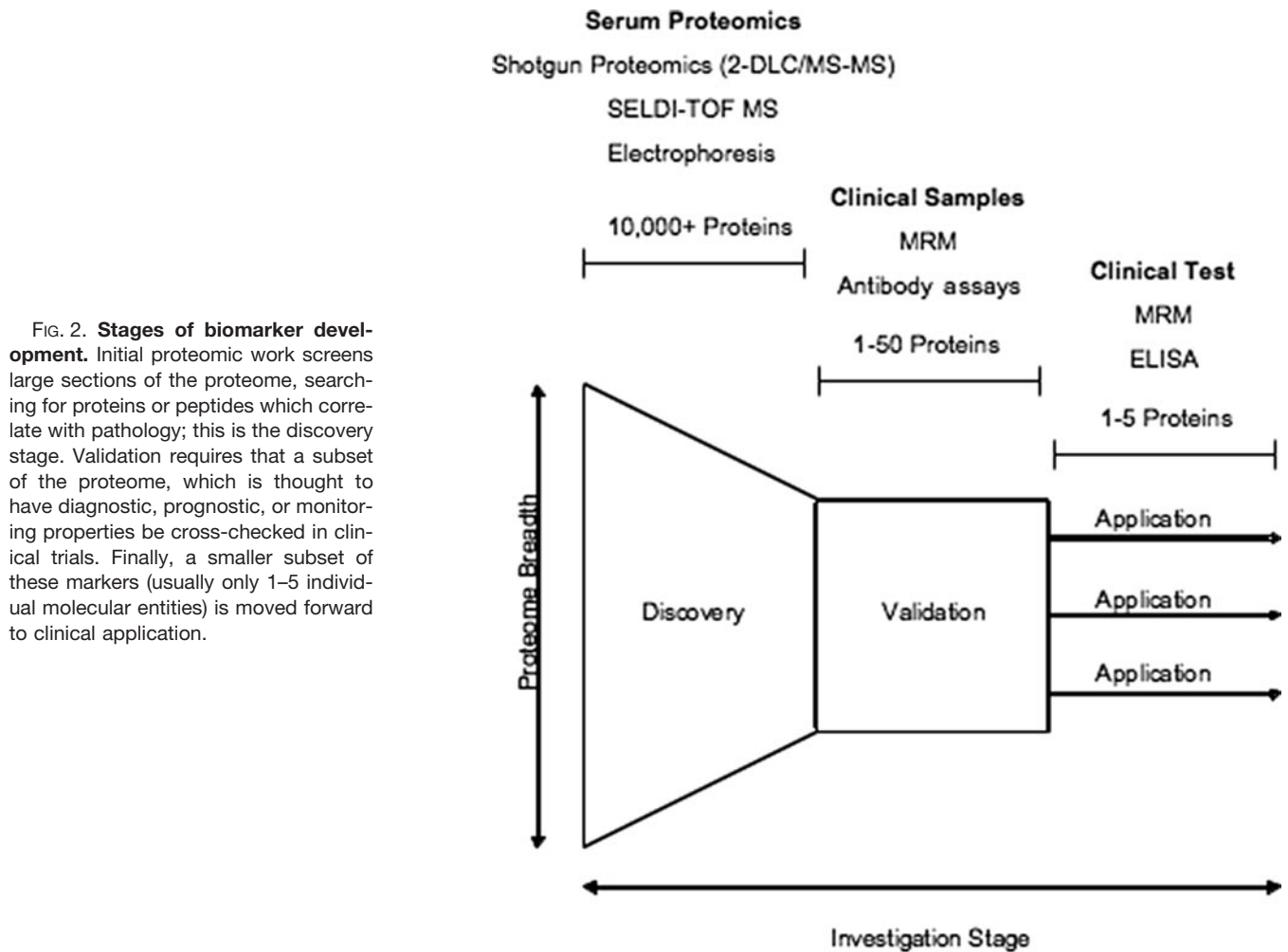


FIG. 2. Stages of biomarker development. Initial proteomic work screens large sections of the proteome, searching for proteins or peptides which correlate with pathology; this is the discovery stage. Validation requires that a subset of the proteome, which is thought to have diagnostic, prognostic, or monitoring properties be cross-checked in clinical trials. Finally, a smaller subset of these markers (usually only 1–5 individual molecular entities) is moved forward to clinical application.

humans have shown changes in several heat shock proteins, which are thought to stabilize components of the sarcomere in pathological conditions (49). This has recently been demonstrated by Lu *et al.* (50), who have been able to show a decrease in TnI and TnT proteolysis in a heat shock protein 27 (Hsp27) overexpression model of I/R, reiterating the proposed role for this protein in protecting the myocardium. The role of stress proteins, particularly Hsp27, highlights the commonality of physiological responses between HF and IHD. Phosphorylation of Hsp27 is a critical response to the early stages of ischemia, and localization of phospho-Hsp27 to the myofilaments also occurs (48), whereas others have shown an increase in Hsp27 phosphorylation during HF (51). Given the protection from ROS-mediated injury thought to be conferred by Hsp27 phosphorylation (52) and the central pathogenic role of ROS in both I/R and HF (53), it is clear that this protein is crucial in the development of, and protection against, I/R injury and HF.

Atherosclerosis—Atherosclerosis, the build up of fatty plaques on arterial walls, is the proximate stimulus for both IHD (after acute plaque rupture) and a contributing factor in

the development of HF (after chronic coronary flow limitation) (54). Proteomic studies of atherosclerosis are hindered by the high degree of heterogeneity present in the vascular tissue, which is the site of the lesion, as well as the heterogeneity of the lesion itself. Despite this, the stable plaque proteome has been mapped (55), and several studies have investigated differential abundance of proteins between atherosclerotic and non-atherosclerotic vessels (55–60). The pathology of atherosclerosis is complex, a fact reflected in the wide range of potential mechanistic proteins that are altered in affected vessels: Hsp27 (56, 57), α -B-crystallin (58), cathepsins (57, 59), tumor necrosis factor receptor (60), peroxiredoxins (56), and many more. There are few identifications which have been confirmed by multiple studies across multiple models (with the exception of cathepsin D) (56, 57, 59, 61), a result certainly affected by the divergent models in use. In recent times, several groups have attempted to examine individual cell types in models of atherosclerosis. Wu *et al.* (62), for example, developed an affinity strategy to specifically examine endothelial proteins in aortic atherosclerosis, using vessel perfusion supplemented with biotin. Labeled proteins were

captured and quantified by LC-MS, and over 80 proteins of endothelial origin were identified as significantly altered in atherosclerotic vessels. The study of atherosclerosis by proteomics is still developing (63), and as more studies are performed and consensus evolves, the mechanistic pathways involved will become clearer.

Biomarker Proteomics—The other major aspect of proteomic studies in CVD relates to biomarker discovery. For the purposes of this review, biomarkers are considered to be proteins or peptides associated with specific diseases or stages thereof, and which, through proteomic techniques, can be detected and used to identify a given pathological state. Note, however, that other biological variables and molecules can be used as markers of cardiovascular states (e.g. cholesterol, blood pressure, etc.) (64) in the more general sense of being “risk predictors” (59). The advantage of protein markers over conventional physiological variables is an increase in both specificity and sensitivity of disease detection and monitoring, with a concurrent increase in information available to the clinician.

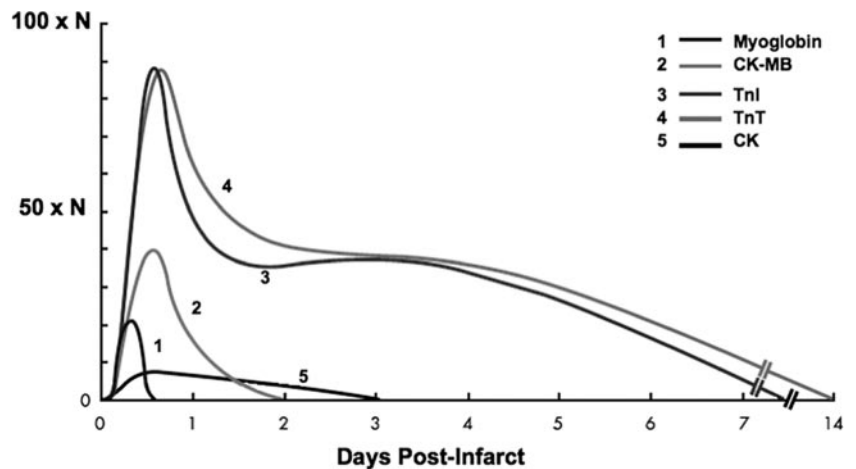
The ultimate aim of locating novel protein or peptide biomarkers of CVD is to broaden the array of clinical tools and tests such that efficiency and accuracy of diagnosis, prognosis, and disease progression monitoring may be improved Fig. 2; (65, 66). For this goal to be reached, potential markers must be validated in trials prior to their routine clinical application. The progression of a potential biomarker toward routine clinical use therefore moves through three stages: identification, validation, and application. Identification involves the initial laboratory discovery of a protein or peptide, which shows evidence of suitability using broad-spectrum proteome screening. The techniques applied here range from 2-DE to “shotgun” mass spectrometry and pattern-based mass spectral approaches, and each has its own benefits and shortcomings. For instance, gel-based discovery experiments will produce easily interpretable visual data but will not detect low abundance or hydrophobic proteins. The second issue is not particularly relevant to biomarker discovery because such studies usually examine human body fluids such as plasma, urine, or cerebrospinal fluid, which obviously contain mainly soluble proteins and peptides. The issue of abundance, or more importantly dynamic range, is far more critical in such studies, particularly for blood fractions, where serum albumin represents ~60% of the total protein and is also known to bind strongly to both blood- and tissue-derived proteins and peptides (67). Similarly, LC-based shotgun workflow will allow direct mass spectrometric linkage, better throughput, analysis of low abundance and hydrophobic proteins, but the data sets are vast, and the interpretation, particularly of quantitative data, requires more complex, informatics-intensive analysis. The choice of the experimental approach is often dictated by the sample type, or in a clinical context, by the ability of the facility to perform the techniques; not all proteomic approaches can be easily translated to clinical work, and not all

are financially feasible on a small scale. Validation of a biomarker requires clinical trials, on as large a scale as possible, in order to assign higher significance to any result that may be found. The precise size of the trial is dependent on the expected variability of the marker in the sample group, in that a highly variable marker will require a much larger sample size in order for any result to reach statistical significance (68, 69).

Finally, after discovery and validation, a biomarker is ready for application in the clinical setting. Once a biomarker has reached this stage, the set of techniques used to detect it changes from high-end, research-focused techniques, to sensitive, specific, and easily performed tests looking for one or a handful of identified markers. As simplicity of use is a factor in the success or failure of biomarkers, the most common technique in this category is enzyme-linked immunosorbent assay (ELISA), which is inexpensive, rapid, and straightforward to use; all desirable qualities of an ideal biomarker test. In more recent times, the implementation of novel techniques to rival standard ELISA-based approaches, such as multiple reaction monitoring (MRM) has occurred, and it is likely that MRM in particular may revolutionize clinical validation and diagnostics using protein/peptide biomarkers (see below). It is important to note here that the proteomics era of biomarker discovery began with the premise of using “pattern-based diagnosis” facilitated by technologies such as surface-enhanced laser desorption ionization MS (SELDI-MS; discussed below) within the clinic itself. This technology, however, has been significantly hampered by technical and philosophical limitations regarding sample handling, data analysis, and validation as well as an understandable reluctance on the part of clinicians to embrace new technology, and it is now anticipated that multiple, independently identified markers (all of which can be tested using conventional ELISA-based approaches) will be the most likely strategies for successful implementation in the clinical environment.

Depending on the specific characteristics of the biomarker (e.g. time and location of detection, biological half-life, mechanistic involvement, etc.), its primary use may be diagnosis or disease monitoring. For example, a powerful diagnostic biomarker would be altered in abundance or specifically modified to a large degree very soon after the initiation of a disease state, allowing for rapid and accurate diagnosis at a stage where clinical intervention is possible. While effective markers of this type give a good indication of the presence or absence of a disease state, they do not necessarily inform the clinician of the degree of injury. In contrast, a marker, which is elevated in proportion to severity of injury, could be used to indicate progression or response to treatment (70). Some markers can address both aspects (e.g. C-reactive protein in acute coronary syndromes (ACS) (71, 72), whereas some are only able to address one or other point (e.g. myoglobin, Tns in ACS) (73, 74). Given this obvious potential and, in several cases, demonstrated benefit (e.g. the widely accepted use of cardiac Tn (cTn) as a marker for IHD) (75–77), protein-based biomarker discovery

FIG. 3. Release profiles of markers of myocardial injury (adapted from Ref. 126). Markers are released from necrotic myocytes following myocardial infarction. Each has a normal range in the blood (N), and diagnosis is made based upon the n -fold level by which clinical measurements exceed this. As shown, serum levels of some markers rise rapidly and remain so for an extended period, whereas others rise more slowly and return to normal range relatively quickly.



remains a focus of intensive study in clinical cardiovascular science.

State of the Art: Cardiovascular Biomarkers

Atherosclerosis and Heart Failure—Although investigations of atherosclerosis by targeted, non-proteomic means have suggested that C-reactive protein, among others, may be serviceable as a biomarker of atherosclerosis (78), direct proteomic studies have been challenged by the local heterogeneity of the plaques. The result of this is that various *in vitro* cell culture models have been favored in proteomic analysis (79). In recent times, one method that seems to have substantial merit for atherosclerosis biomarker discovery is the identification of proteins secreted by atherosclerotic plaque cultured *in vitro* (80). Such proteins have a high probability of *in vivo* release into the circulation and hence potential detection in the blood of patients with underlying plaque. A similar approach examined the culture supernatants of “foam” cells, stimulated by either normal or oxidized low density lipoprotein, and identified several proteins with altered abundance from functional groups implicated in atherosclerosis (59). Alternatively, Tabibiazar *et al.* (81) have suggested that multiple inflammatory markers in human serum could be utilized to predict the presence of atherosclerosis, as single markers alone have poor specificity and sensitivity. This group used protein microarrays to monitor the abundance of 30 inflammatory markers in the serum of ApoE-deficient and wild-type mice and predicted that a subset of these might have predictive utility in human studies. As is the case for mechanistically oriented studies of atherosclerosis, further and more coordinated work will lead to better direction and firmer results (82).

With regard to HF, the only biomarker in common clinical use for diagnosis and monitoring is B-type natriuretic peptide (BNP) or a fragment of the N terminus of BNP pro-hormone, N-terminal fragment of B-type natriuretic peptide pro-hormone (NT-proBNP) (83). Each of these is released from myocardium undergoing wall stress, a common occurrence in HF as well as in IHD (84, 85). This overlap limits the specificity and

therefore the applicability of these markers, which is compounded by the range of other conditions that affect BNP/NT-proBNP levels (86), as well as disease-induced post-translational modification that may influence their success as markers. Despite this, BNP has been shown to have prognostic value in a clinical setting (87), and there are no significantly superior alternative biomarkers of HF among the available suite (88–90).

Ischemic Heart Disease—Possibly the most widely applied biomarker tests are those relating to acute myocardial ischemia. These markers are released from myocytes as a consequence of injury and can be detected in circulating blood. Unfortunately, most reasonably specific markers (including the Tns and CK, as discussed below) are released after the onset of significant necrosis, and at which stage it is impossible to salvage dead myocytes. There is encouraging evidence, however, that markers may exist which are released upon initiation of ischemia alone (91). If true, such markers would offer the opportunity for specific recognition of IHD at a time point where salvage of the myocardium by timely reperfusion is possible and before permanent tissue damage occurs. Such a marker would have significant health benefits in reducing the number of patients turned away from critical care or immediate response centers because of ambiguous results from currently available diagnostic tests, and who later die of myocardial infarct. Conversely, such a specific marker would also reduce healthcare costs for patients presenting with CVD-associated symptoms (e.g. chest pain, radiating pain down left arm), with ambiguous diagnostic results, but which resolve as being non-cardiovascular in origin.

As illustrated in Fig. 3, each currently utilized clinical marker of myocardial infarction has a very specific release profile, meaning that different markers are available for detection in the blood after varying periods of injury and remain available for varying periods post-insult. The choice of biomarker is therefore a critical factor in efficiently achieving an accurate diagnosis. Here, we examine individual markers and discuss their utility in the clinical environment.

Myoglobin—Myoglobin can first be detected in blood ~2 h after clinical presentation, *i.e.* onset of chest pain. This rapid rise means that myoglobin is one of the first detectable markers of injury. As with other markers, however, the primary drawback of myoglobin is a lack of specificity; it is also released from skeletal muscle upon injury, limiting the utility of this marker as a positive predictor for myocardial ischemia. Myoglobin also has a relatively short half-life in blood, with peak concentrations reached ~6 h post-infarct and returning to base-line levels over the following 18 h. This means that maximum predictive value is only extracted from myoglobin tests within ~6 h of experiencing chest pain; beyond this stage, other markers are more appropriate (92). Myoglobin is also relatively insensitive *versus* alternatives such as cTn (comprising TnI and TnT) with current assay technology, which, when combined with the above limitations, renders the value of myoglobin tests marginal at best. Although it may have future application as part of a panel of biomarkers of IHD (93), in most applications myoglobin has been superseded by the use of cTn.

Creatine Kinase—Creatine kinase (CK) can be measured as total CK or as a combination of the CK muscle and/or brain isoforms (CK-MM, BB, and MB). Measurement of CK-MB has been an integral part of IHD tests for many years, though CK-MB-based diagnostics are not without flaws. As in the case of myoglobin, CK-MB suffers from a lack of specificity for cardiac injury; though the MB isoform is undoubtedly significantly more cardiac-specific than either of the other isoforms or indeed total CK. Creatine kinase is released from injured myocytes ~2 h post-infarct and remains detectable in blood for up to 48 h (92) (Fig. 3). CK-MB therefore, like myoglobin, has application in the early detection of infarction given its comparatively rapid rise in blood concentration, although it is suggested that other more powerful markers (in particular cTn) may be able to perform the same role. This is the crux of the issue regarding the choice of CK-MB as a primary marker for IHD, in that cTn can arguably provide a better diagnostic tool regardless of time point (see Fig. 3). The possible exception to this is when diagnosing secondary infarction, as Tn levels remain elevated for 10 or more days after index necrosis (depending on Tn form), thus limiting their capacity to distinguish between two separate, yet closely spaced, ischemic events. In this situation, CK-MB levels, which return to base line over a much shorter period, can elevate once more in response to re-infarction, and a significant alteration to the decline profile can be observed. However, there is not unanimous agreement among clinical cardiologists as to whether this limitation of cTn is simply an artifact of particular assay conditions.

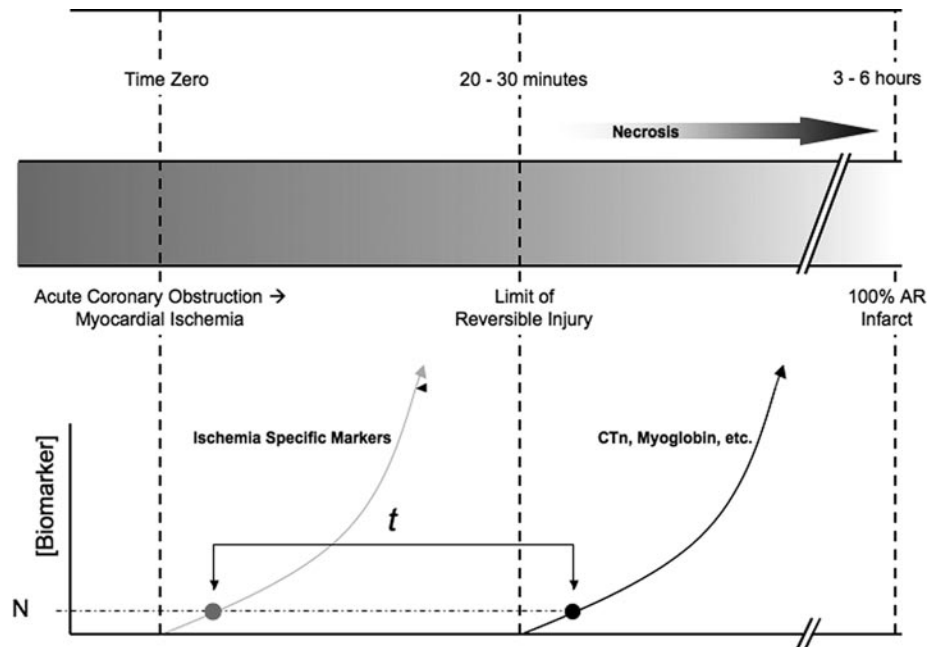
B-Type Natriuretic Peptide—B-type natriuretic peptide (BNP) is released from myocytes in response to increases in cardiac wall stress and is commonly used as a biomarker of HF (85). IHD leading to necrosis is also capable of producing increased cardiac wall stress and leading to BNP release. This

provides a rationale for using BNP levels as a diagnostic test for acute coronary syndromes; however, BNP-based diagnostics are also poorly specific as the primary stimulus for BNP release, cardiac wall stress, results from many other conditions in addition to IHD: right-sided heart failure, ventricular hypertrophy, and pulmonary embolism, to name but a few (75, 84, 92). In addition to this lack of specificity, BNP levels are subject to enormous biological variability (75). This makes it difficult to assign upper and lower limits to exclude or diagnose disease and, consequently, difficult to use BNP levels as a routine test, be it for diagnostic, prognostic, or monitoring purposes. Work performed to test the efficiency of combining BNP and cTn to detect and monitor IHD yielded better sensitivity and specificity (94, 95), strengthening the argument for a combinatorial approach to biomarker use for clinical cardiovascular diseases.

Cardiac Troponins—Cardiac troponins are the current gold standard markers for myocardial necrosis (76, 77). cTn exists in three separate forms (TnC, TnI, and TnT), of which only TnT and TnI are used as markers of IHD; TnC is generally not used due to low cardiac specificity. Detectable 2–3 h after the onset of symptoms, cTns are extremely sensitive, highly cardiac specific, and arguably as effective in early stage necrosis as CK-MB or myoglobin under the correct assay conditions and detection limits (75). As mentioned earlier, the slow removal of cTns from post-infarct circulation makes them somewhat less useful in situations of re-infarction, though studies have been performed in which re-infarction is recognized from cTn tests with an accuracy equal to that of CK-MB tests (96). It is clinically important to note that cTn are diagnostic of myocardial damage but do not specifically indicate IHD. Many other pathologies can produce cardiac injury and cause release of cTn from the myocardium, and this must be taken into account (75, 97–99). In addition, cTns are subject to extensive PTM and have been shown to exist in modified forms in patients with ACS, complicating diagnostic quantification. Though cTns are undoubtedly currently the best performing clinical biomarkers, these points are once more strong justification for the use of panels of biomarkers to reduce clinical uncertainty, a subject we will return to below.

Ischemia-specific Markers—One significant shortcoming of the current list of biomarkers is that all require a level of myocardial necrosis to prompt their release from cells before they can be detected. A biomarker not subject to this requirement, and which could be detected before the onset of significant necrosis, would be a vast improvement upon those in current use. As shown in Fig. 4, such a marker would allow for earlier intervention, greater salvage of the myocardial area at risk, and ultimately, decreased mortality from acute coronary syndromes. Therefore, identification of an early onset, ischemia-specific biomarker is a focus of intensive research in cardiovascular proteomics. Such a marker could be the result of several events at the cellular level; firstly, ischemia could lead to the release of specific proteins or peptides from myo-

FIG. 4. Release profiles of current and theoretical biomarkers of IHD. In humans, necrosis begins some 20–30 min after the onset of ischemia. It is only after this time that current commonly used biomarkers are released from myocytes, and some period of time after this point that levels rise sufficiently to provide diagnostic confidence (above the normal range, N). There may exist other, equally specific markers, which are released immediately following initiation of ischemia. Assuming similar N values, these markers will increase the time (t) available to the clinician to diagnose IHD prior to the onset of necrosis.



cytes prior to necrosis, through, for example, altered membrane permeability; and secondly, ischemia could result in modification of components of the pre-existing blood proteome/peptidome without specific release of proteins from the myocytes themselves. A third possibility is the release of proteases from myocytes under ischemic stress, resulting in both the appearances of new proteins within the circulation and modification by degradation of those proteins already contained within the circulating blood. While any of these scenarios are possible, a major issue for discovery of an early biomarker of ischemia is whether the alteration to the protein composition of the circulation would be sufficient to be detected using currently available technology for screening the human plasma proteome (Fig. 4).

Currently, the only commercially available ischemia-specific marker is IMA. In this instance, albumin is N-terminally modified following the onset of myocardial ischemia. The effect of this PTM (although the exact nature of it is yet to be determined) is to decrease the ability of albumin to bind transition metals, allowing for quantification of modified albumin, and hence the degree of ischemia, with a simple assay (100–104). Unfortunately, as is often the case with blood-based biomarkers, IMA is poorly specific with regard to IHD (73), though this is balanced somewhat by the benefit of early indication, rather than firm diagnosis. To optimally contribute to the diagnostic process, IMA has been paired with conventional ACS biomarkers (cTn) to raise specificity, but unfortunately at the cost of earlier diagnosis (101). Other novel ischemia-specific biomarkers are currently in development (e.g. choline and glycogen phosphorylase isoforms) (105), but at the time of writing none are nearing clinical application.

The proteomic screening of pre-necrotic stages of myocardial I/R injury has enormous potential to allow early diagnosis and hence intervention in IHD. As shown in Fig. 4, there is a well defined period during which ischemia is potentially symptomatic, but prior to the onset of necrosis. During this period the ability of the clinician to accurately diagnose IHD is limited, and therefore a significant proportion of patients with IHD are not recognized and a corresponding proportion without IHD are misdiagnosed in the opposite fashion.

Limitations of Current Cardiovascular Biomarker Technology—To “discover” a novel biomarker it is necessary to screen the entire proteome of the body compartment in question and identify proteins or peptides that change, reproducibly, in concert with disease state. Although it is not an absolute necessity to search *only* within the compartment directly related to the disease/organ in question, the likelihood of locating relevant and specific proteomic changes is much greater if one does. For example, markers of renal injury may be found anywhere – tears, saliva, blood – as long as there exists a link, however tortuous, between the kidneys and the site of detection such that disease may be reliably detected and/or monitored. With increasing separation in physical and functional space, however, the number of links between two systems will decrease. This is why markers of renal injury, for example, tend to be found in compartments directly relating to the kidneys (e.g. urine); those of the brain, to the brain (e.g. cerebrospinal fluid), and so on. When searching for biomarkers of CVD it is therefore sensible to begin by examining the blood. An ideal marker should, for maximum utility, require minimal clinical invasion; therefore blood and other body fluids remain the typical compartments for study (68, 106, 107). Tissue biopsies, in contrast, although certainly a source of

disease information, are not preferred as biomarker sources for most organ diseases and are completely impractical in cardiac disease. Unfortunately, however, as directly related to the cardiovascular system as the blood may be, it is equally related to every other organ in the body. Therefore, blood-borne biomarkers for CVD must be extremely specific, and, as outlined above, not all currently satisfy this requirement. Some, as in the case of cTn, reach very high levels of specificity, whereas others, for example, myoglobin, do not (75). Blood-borne markers for other systems are naturally subject to the same requirements for specificity, but in the event that a given marker falls short these systems often have easily accessible alternative, equally specific sources of biomarkers; the renal system, for example, can offer urine as a source of potential biomarkers and loses no degree of specificity in doing so. The only corresponding, specific biomarker reservoir for the cardiovascular system is the pericardial fluid, which will be discussed below. In addition to specificity, there are questions of reproducibility when screening the blood proteome – the methods used to remove cells, coagulate (if using serum), and inactivate proteases will all affect the protein/peptide profile of the final sample (108).

When attempting to detect potential blood-borne biomarkers during the discovery stages of a cardiovascular clinical proteomics workflow, one comes up against a second key problem, in addition to that associated with specificity. The presence of highly abundant serum/plasma proteins, which extend the dynamic range of the sample by several orders of magnitude, masking less abundant proteins, presents the single most important challenge, which biomarker proteomics must overcome. In most cases these abundant proteins do not contain significant disease information and are regarded as “interference”. The level of this interference is demonstrated by studies in which an analysis of plasma from ACS patients revealed only five differentially abundant proteins, all of which were highly abundant plasma proteins (109). These confounding proteins prevent detection and analysis of low level proteins, for example tissue leakage proteins, which are often those of interest to the study. This problem can be avoided to some extent through pre-fractionation of samples prior to analysis in order to remove highly abundant proteins and simplify subsequent investigation. For instance, there are well characterized methods to deplete serum samples of immunoglobulins and serum albumin (110, 111). Unfortunately, these depletion techniques can also lead to inaccuracies; some of the most abundant blood proteins (e.g. albumin) are “sticky” and bind other, less abundant peptides and proteins, which are then removed from the analytical pool along with the unwanted protein. Again, as the proteins, which show most promise as markers are in low copy number; biomarker discovery rates can be significantly decreased in this way. Investigators have analyzed both the “albuminome”, the subset of proteins bound to serum albumin after depletion with anti-albumin antibody (67), as well as the “plasma protein-

associated proteome” where over 200 proteins are identified as binding to a selection of abundant plasma proteins (apolipoprotein, IgM, IgA, and albumin); approximately three quarters of which are not previously identified in the plasma proteome (112). These problems are overcome to some degree by the parallel analysis of proteins bound to the depletion agent as well as the depleted sample. It should also be noted that parallel use of many different proteomic approaches (for example, as used in the Plasma Proteome Project) (108) is able to increase depth of proteome coverage and limit the effect of the wide abundance range of plasma proteins.

For the identification of cardiovascular biomarkers, particularly those associated with I/R injury, it is possible to model biomarker release in *ex vivo* perfusion models, which dispense with blood altogether (113) and then search for the identified released proteins and peptides specifically in human plasma using alternate strategies such as ELISA-based or Western blotting assays. In this approach, hearts are excised from euthanized animals and perfused with non-blood buffers. The hearts can then be subjected to various ischemic insults, reperfused, or allowed to proceed to full infarct in a controlled environment where functional (hemodynamic) data and myocardial recovery post-injury can also be monitored. Tissue and the circulating buffer perfusate can be examined for proteomic differences. The advantage of this method is that the non-recirculating buffer removes the vast majority of abundant blood proteins during base-line (controlled) perfusion such that any proteins or peptides released during injury have a much better chance of being detected using standard proteomics methodologies (Fig. 5). The major drawback with perfusion-based approaches to marker discovery is that they rely on the animal model being an accurate representation of human disease processes, at least for the protein/peptide of interest. As we have discussed previously for TnI proteolysis and MLC-2 modification in IHD, this is not always the case; however, the advantages of significantly reducing the plasma proteome “haystack” to identify biomarker “needles” are philosophically very sound.

Given the obstacles to comprehensive and accurate proteomic profiling of blood and blood products, those seeking to identify biomarkers of CVD, and in particular diseases relating directly to the myocardium such as HF or IHD, have also begun to examine pericardial fluid. Tambara *et al.* (114) have shown an increase in pericardial levels of heart type fatty acid-binding protein (H-FABP; another known marker of myocardial necrosis) in the period immediately following myocardial ischemia in humans undergoing cardiac surgery and, importantly, have also shown that this increase is not reflected in serum levels of H-FABP over the same period. This suggests that the pericardial fluid does indeed have its own distinct proteome and may potentially contain useful markers of cardiac pathology. Despite this, the pericardial fluid proteome remains mostly unstudied. Several groups have performed targeted investigations looking at one or a

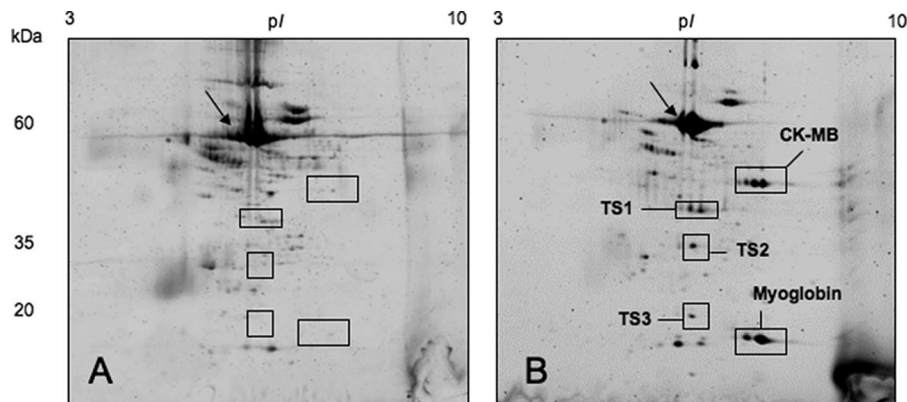


FIG. 5. **Comparative 2-DE gel profiles of buffer perfusate from a rabbit model of I/R injury.** *A*, perfusate proteins collected from baseline (control, no ischemia) perfusion. *B*, perfusate proteins collected between 25–30 min of reperfusion following 60-min ischemia (infarction with necrosis model). Proteins highlighted in *boxes* are derived from myocardial tissue and include known markers such as CK-MB and myoglobin. While some blood-derived proteins remain (e.g. serum albumin, highlighted with *arrow*), their relative amount is greatly reduced, allowing for the easy identification of TS proteins and peptides. TS, tissue-specific.

few likely candidate proteins and have indeed found significant changes as a result of various cardiac pathologies (115, 116), but a true proteomic profile has not yet been obtained. The fact that it has been demonstrated that the pericardial fluid displays protein changes, which reflect disease state is enough to render it a potentially useful reservoir for biomarkers and worthy of further research in this respect (117).

New Technologies: Pattern-based Diagnostics—Proteomic investigations, in particular those aimed toward biomarker discovery, have made extensive use of chromatographic separation of extremely complex mixtures of peptides and proteins in order to maximize detection of any one component (118). Reverse phase liquid chromatography followed by tandem MS (RPLC-MS/MS) is the basic component of most shotgun workflows, and, as mass spectrometric and chromatographic technology improves, the capability of these techniques continues to increase. In addition to these steady technological improvements, there has been a shift in recent years toward biomarker discovery using mass spectrometric pattern-based approaches, a particularly attractive option for two reasons: firstly, there is greatly enhanced sample throughput, and secondly, disease “patterns” are likely to be highly specific because they are comprised of several individual markers. Conversely, the impact of the technology has been reduced clinically by the need to purchase specialized instrumentation and software for specific tests that are not yet fully approved for clinical use, the requirement for additional training for clinical staff, and a general field-wide discussion regarding the strengths and limitations of the technology and the overall approach to sample handling, discovery, and validation using proteomic approaches. At the current time, it is likely that the technologies will be primarily utilized for discovery rather than as diagnostic instrumentation that can be implemented clinically. Discoveries based on MS patterns are now being interrogated to discern the identities of the individ-

ual markers, such that standard testing based on those suites of markers can be put on trial and implemented.

Primary among the technologies utilized for pattern-based biomarker discovery are SELDI-TOF MS and MRM. SELDI-MS involves sampling a fraction of the proteome through interaction chemistry using antibodies, ionic interactions, and hydrophobicity, among many others (119). These interactions are used to bind proteins to a target, which is subsequently analyzed by MS. Many thousands of protein/peptide binding assays can be performed and quantified per run to yield a sample MS “signature”, which, through computational comparison with signatures from healthy and other disease states, can be used in a diagnostic capacity. Alternatively, if the proteome area of interest is known in advance, a signature for a specific subset of proteins can be generated and used in the same way, a principle validated for cTnI during acute myocardial infarction (120). The major benefits of SELDI-MS are its disposal with the need for primary sequence data and the reduction in complexity of the proteome, expediting, and simplifying the overall process (121); however sample handling remains a critical aspect in the reproducibility of the pattern, and most studies require validation of the individual components of the signature (122). MRM has significantly greater potential as a diagnostic approach, particularly in validation because it relies on sampling a directed subset of the proteome to reduce analytical complexity and is based on previously identified markers. MRM approaches monitor a selection of mass spectrometric m/z “nodes” where characteristic ions (the potential “biomarkers”) are known to appear (123). Both SELDI and MRM can be combined with quantitation techniques to obtain greater confidence in the generated patterns and allow some degree of gradation of diagnosis and/or monitoring. Pattern-based MS technology has strong potential for use in IHD and HF. In addition to classical biomarker assays, mass spectral patterns provide another route to rapid and precise diagnosis. While a truly

effective pattern-based tool requires a considerable amount of pre-clinical work to be confident in its ability to reflect pathological states, this is no less than is the case for conventional biomarkers. Therefore, it is the opinion of many scientists and clinicians that proteomic or peptidomic patterns may ultimately represent the next stage in disease diagnosis (124, 125).

CONCLUSIONS

Proteomics has contributed significantly to the field of clinical cardiovascular science, both through exploring mechanisms of disease generation and progression as well as by allowing those same processes to be identified, and thus also treated, more efficiently. The protein networks involved in IHD and HF are by no means completely characterized, but proteomics has the potential to reveal those proteins that are associated with pathogenesis. With a greater understanding of information flow in pathogenic situations there will be an increased opportunity for interventions to limit the impact of disease, and this is an area where proteomics has enormous potential. In addition to the mechanistic contributions of proteomic science, continuing improvements in technical ability (for example, in the field of mass spectrometry) will lead to more effective biomarker discovery and application; in particular, we are hopeful that these developments coupled with proteomic research into early-stage ischemic heart disease will allow identification of ischemia at a point in time where the myocardium can be largely salvaged prior to cell death. This will in turn ultimately lead to increased efficiency of diagnosis and/or monitoring of treatment, which could dramatically increase the ability of the clinician to recognize cardiovascular disease states at a relatively benign stage, with a concurrent decrease in morbidity.

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