

## Topical Review

# Candidate-based proteomics in the search for biomarkers of cardiovascular disease

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The key concept of proteomics (looking at many proteins at once) opens new avenues in the search for clinically useful biomarkers of disease, treatment response and ageing. As the number of proteins that can be detected in plasma or serum (the primary clinical diagnostic samples) increases towards 1000, a paradoxical decline has occurred in the number of new protein markers approved for diagnostic use in clinical laboratories. This review explores the limitations of current proteomics protein discovery platforms, and proposes an alternative approach, applicable to a range of biological/physiological problems, in which quantitative mass spectrometric methods developed for analytical chemistry are employed to measure limited sets of candidate markers in large sets of clinical samples. A set of 177 candidate biomarker proteins with reported associations to cardiovascular disease and stroke are presented as a starting point for such a 'directed proteomics' approach.

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Proteomics has been defined from the biochemist's viewpoint (in a remark by Kenneth Mann) as the study of more than one protein at a time, a perspective that recognizes the importance of complex relationships between the functional parts of living systems while resisting the temptation to insist on a genome-style complete (and perhaps unattainable) description at the protein level. While the technologies of proteomics have made rapid strides in recent years, providing tools that have been applied to many disease processes, there is a conspicuous lack of important disease markers discovered through proteomics and now established in the clinic. In fact if the rate of new plasma diagnostic protein markers is examined over the last decade, it has actually declined from one to two per year to near zero today (Anderson & Anderson, 2002). The reasons behind this paradox deserve study because the potential importance of accessible protein biomarkers of both normal and abnormal physiology is so great, particularly if we can believe the attractive but unproven hypothesis that all abnormal physiological states leave some specific fingerprint in the composition of circulating proteins. Evidence for this hypothesis, most recently in the field of cancer detection (Petricoin *et al.* 2002), has been accumulating for many years in the related fields of metabolite analysis (Jellum *et al.* 1981) and clinical chemistry (Robertson *et al.* 1980). These studies add support to the general statistical argument that a panel

of independent disease-related proteins considered in the aggregate should be less prone to the influence of genetic and environmental 'noise' than is the level of a single marker protein. The heterogeneity of disease processes, and the genetic differences between individuals in the human population, both tend to obscure what might otherwise be clear disease associations. However, if there are multiple markers affected by the disease which are not strongly correlated with one another, then a composite index combining these markers may provide a much more robust indication of disease. In measuring the acute phase response, for example, a composite index summarizing a panel of weak acute phase reactants (Doherty *et al.* 1998) can provide a more robust indicator of inflammation than a single marker (e.g. C-reactive protein (CRP) or serum amyloid A). Similarly the relative risk of coronary heart disease is better predicted (Rifai & Ridker, 2003) by CRP and low-density lipoprotein (LDL)-cholesterol together than by either alone (Fig. 1, replotted from published data: Rifai & Ridker, 2003). More sophisticated multiplex panels have emerged from work with microarrays. One such example is the Netherlands breast cancer study (van't Veer *et al.* 2002), which sought to distinguish between patients with the same stage of disease but different response to treatment and overall outcome. The success of this initial study motivated a more extensive independent follow-up study involving 295 patients (van de Vijver *et al.* 2002)

which led to a nationwide clinical trial in the Netherlands in which gene expression profiles for 70 classifier genes are being collected on all breast cancer patients and used as an adjunct to classical clinical staging. The belief that this phenomenon will be general for both proteins and mRNA, and that combinations of markers can be found that will identify and stage a wide range of diseases with useful specificity and sensitivity, is among the most important hypotheses of current biomedical research.

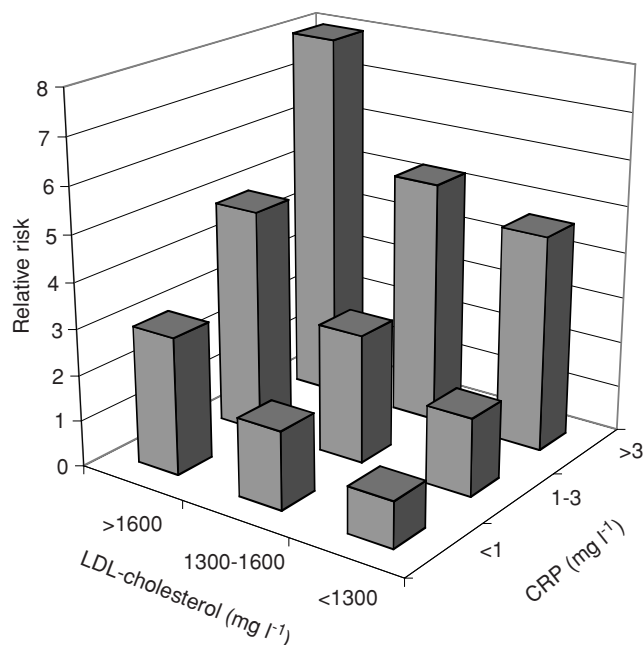
The difficulty of finding and using new biomarkers in the blood, even given the impressive advances in proteomics technologies, becomes clear when we compare the characteristics of the plasma proteome with the capabilities of current proteome analysis strategies and technology platforms. An exploration of this juxtaposition, set out in the following sections, provides the basis for an alternative candidate-based (targeted) approach proposed in the remainder of the paper.

### Challenges of the plasma proteome

Plasma, which (together with its close cognate serum) is the primary biochemically useful clinical specimen, comprises the largest and deepest version of the human proteome. This makes it the most difficult sample to work with in proteomics, despite the relatively good behaviour (i.e. solubility) of its protein components. The daunting size of the plasma proteome is a reflection of the sheer number

of different proteins to be detected. A rough calculation of this number can be made as follows. (1) Assume that 10% of the  $\sim 30\,000$  genes encode secreted proteins, that each of these is made in an average of three splice forms, that two cleaved versions of each exist, and that there are an average of five post-translational modifications for each protein (a low estimate given the extreme carbohydrate microheterogeneity of most major plasma proteins). Since all these events can occur independently of the others, we obtain  $3000 \times 3 \times 2 \times 5 = 90\,000$  different secreted molecules. (2) Assume that all the non-secreted human proteins and their various modified forms are released into plasma at some low level as a result of cell turnover in the tissues. Using levels of modification similar to the secreted proteins, we obtain a further 810 000 protein species present at low levels. (3) Finally, assume that there are  $\sim 10\,000\,000$  distinct clonal immunoglobulin sequences present in plasma reflecting the immune history of the individual. The sum of these admittedly rough estimates is  $> 10^6$  different molecules representing products of all  $\sim 30\,000$  genes: in other words, plasma is the largest version of the human proteome in one sample. Proteomics technologies can typically resolve  $\sim 100$  different species per dimension of separation, indicating that 3 or more perfectly independent separative dimensions would be required, or more probably 4–5 dimensions of realistically implementable technology.

The enormous 'depth' of the plasma proteome is a reflection of the dynamic range (difference between the highest and lowest concentration) over which proteins must be detected. Approximately half of the total protein mass in plasma is accounted for by one protein (albumin, present at  $\sim 55\,000\,000\,000\text{ pg ml}^{-1}$ ), while roughly 10 proteins together make up 90% of the total. At the other end of the concentration histogram are the cytokines, such as interleukin-6 (IL-6), which is normally present at  $1\text{--}5\text{ pg ml}^{-1}$ . The difference in concentration between albumin and IL-6 is thus  $\sim 10^{10}$ . This range, of course, covers the proteins we know and consider useful as markers today, and ignores potentially valuable markers to be found in the future at even lower concentrations. The fact that we know anything about the concentrations of these proteins, and hence have been able to use them as biomarkers, is due to the power of specific protein tests, typically immunoassays of one protein at a time, and not to proteomics as currently defined, where currently technology is limited to a dynamic range of  $10^3\text{--}10^4$  (see Fig. 2).



**Figure 1**

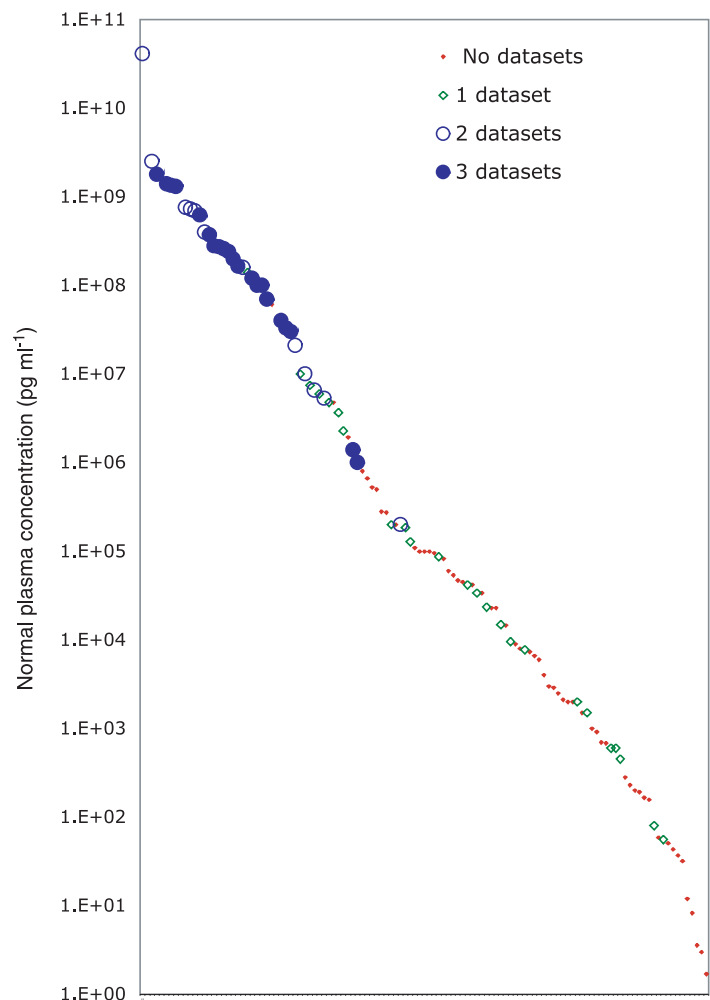
Data replotted from Rifai & Ridker, 2003 showing the improved discrimination of relative cardiovascular disease risk when two different markers (in this case LDL-cholesterol and C-reactive protein) are considered jointly.

### Proteomic strategies for the discovery and validation of biomarkers in plasma

Given the analytical challenges inherent in the plasma proteome, what practical strategies exist for finding and confirming protein biomarkers? The problem can be

approached from two opposite directions: (1) complete analysis (to see all differences) and (2) targeted analysis (to measure one or more hypothesis-generated candidates). The advantages of complete analysis, if it is possible, are substantial. Complete analysis would allow the direct selection of optimal biomarker proteins at the outset, thus skipping over what is currently a very long and laborious iterative process. Not surprisingly, progress towards complete analysis has been the focus of most proteomics research for the past decade. The number of proteins detectable in plasma has risen from 40 in 1975 (Anderson & Anderson, 1977) to 300–1000 reported in various recent studies (Adkins *et al.* 2002; Pieper *et al.* 2003a; Tirumalai *et al.* 2003). The latter datasets have been combined (Anderson *et al.* 2004b) to generate a non-redundant set of 1173 proteins, which revealed surprisingly small commonality between the results of these three different proteomics platforms (respectively multidimensional chromatography of proteins followed by 2-D electrophoresis and mass spectrometry (MS) identification of resolved proteins; tryptic digestion and multidimensional chromatography of peptides

followed by MS identification; and tryptic digestion and multidimensional chromatography of peptides from low-molecular weight plasma components followed by MS identification). When these datasets are searched for a group of candidate disease markers (the cardiovascular candidates described below) for which plasma concentration normal values exist, the result illustrates the limited sensitivity of the platforms as a means of complete plasma proteome analysis (Fig. 2). Most proteins in the top 3 logs of the concentration distribution are detected by two or three of the three platforms, a fair proportion of the proteins in the middle two logs are seen by at least one of the platforms, and very few of the proteins in the bottom 5 logs are detected by any of the three. Thus it appears that current proteomics technology is unlikely to be able to provide a complete analysis of the most relevant diagnostic samples (e.g. serum and plasma). An additional important feature of this plot is that the candidate proteins show a smooth distribution between  $10$  and  $10^9$   $\text{pg ml}^{-1}$ , demonstrating that presumed disease relationships appear to occur independently of a protein's plasma concentration. In particular there does



**Figure 2**

A plot in which normal plasma concentrations for 115 proteins from Table 1 (distributed along the X-axis but unlabelled because of legibility limitations) are plotted on a log scale ( $\text{pg ml}^{-1}$  along the Y-axis). The proteins are sorted by abundance to reveal the smooth distribution across > 10 logs of concentration. Each protein is represented by a symbol that indicates in how many of three proteomics datasets (see text) it was detected.

not seem to be a bias towards either very low abundance proteins (e.g. cytokines) or high abundance molecules. Since plasma concentration was not a criterion in the selection of these proteins (just a relationship with cardiovascular disease or stroke), this observation is probably meaningful.

Targeted analysis, which emerged as a means of searching for disease marker associations in the 1950s (in the form of enzyme assays), has a longer history than proteomics (which emerged in the form of 2-D electrophoresis in the mid-1970s), and has produced most of the protein markers now in diagnostic use. Typically a researcher interested in a specific protein develops hypotheses regarding a specific disease, and arranges to apply a lab bench assay to sets of samples from patients and controls. The specific assays involved are usually immunoassays, which, because of the great specificity of antibodies, are often able to detect proteins in plasma at much lower concentrations than current proteomics platforms. While this approach adheres to the conventions of hypothesis-driven research (and is thus fundable through grants), it has a substantial weakness in the poor probability of success when one marker is tested in one disease at a time: there are, as indicated, at least tens of thousands of candidate protein forms, and at least hundreds of disease entities. Even if it were the case that there is one protein capable of serving as a robust marker of each disease state, this method will take a long time to find them, and unfortunately it will take as much effort to find the last such marker as it took to find the first. More discouraging yet is the fact that any disease state in which several markers need to be considered together to produce an accurate result (i.e. a multiplex panel) would represent an enormous combinatorial discovery problem: since the experimental assays are typically developed in separate laboratories, bringing them together for application as a prototype panel is an organizational challenge, compounded by the increased sample requirement of multiple separate assays.

Thus both the complete and targeted analytical approaches have important limitations (sensitivity and mono-analyte focus, respectively) that diminish the output of novel disease marker proteins. This situation has led in recent years to consideration of hybrid approaches, in which a set of preselected proteins could be measured at high sensitivity. By focusing on a limited number of candidate biomarker proteins, assay technologies providing higher sensitivity and dynamic range than current proteomics could be used. By looking at multiple proteins, instead of one, the odds of finding useful disease associations, and effective panels, would be increased. The odds can be further improved through intelligent selection of candidate markers: here there is an opportunity to make use of information from many sources in addition

to proteomics: expression microarray data suggesting tissue-specific or disease-altered synthesis of specific proteins, relationships of proteins to disease pathways, and classical biochemistry. Such a hybrid approach, combining the multiprotein view of proteomics and the advantages of targeted specific assays can be termed targeted proteomics.

### Technology platforms for targeted proteomics of candidate markers

The central technical issue in targeted proteomics is how best to measure a limited set of proteins in complex samples such as plasma. Two broad strategies are developing: miniaturized, multiplexed immunoassays and quantitative mass spectrometry. The former approach, which includes antibody arrays in both planar and particle suspension formats (recently reviewed by Joos (Joos *et al.* 2002) and a review in this series), has the advantage that immunoassays are well-understood, sensitive and specific. Antibody arrays are limited, however, by the availability of suitable antibodies, and this has proved to be a critical bottleneck for the development of immunoassays for new marker content. While a single research immunoassay costs less to assemble than the \$2–4 million required for a commercial diagnostic test, each additional new marker assay costs the same again as the first, typically requiring development of two different high-affinity antibodies. It thus appears that substantial time will be required to generate large sets of new immunoassays to candidate markers, and that an alternative approach based on quantitative mass spectrometry may serve to evaluate candidate biomarkers prior to investment in immunoassays. Here I focus on the emerging MS methodologies for specific protein quantification.

Mass spectrometry is widely used for the quantitative measurement of specific small molecules (e.g. drugs (Streit *et al.* 2002, 2004), drug metabolites (Kostiainen *et al.* 2003), hormones (Tai *et al.* 2004), and pesticides (Sannino *et al.* 2004)), with excellent precision (Tai *et al.* 2004) and very high throughput (Bakhtiar *et al.* 2002; Deng *et al.* 2002). In these methods, a sample is typically subjected to some form of high-throughput pre-fractionation (e.g. solid phase extraction; SPE) followed by a rapid reversed-phase chromatography separation, and the resulting output stream is introduced through an ionizing spray interface into a triple-quadrupole MS (TQMS). Within the MS, the first mass analyser (MS1) is set to pass the *parent molecule* (the 'analyte'), rejecting components of other mass-to-charge ratios ( $m/z$ ). The analyte is then fragmented in a collision chamber and passed to a second mass analyser (MS2) set to pass a known *specific fragment*. This two-stage selection of parent and fragment ions (selected reaction monitoring; SRM) affords

great specificity, with the result that the detected signal usually traces a peak in the chromatogram at the expected retention time corresponding to the selected analyte. Integrating this peak gives a measure of the quantity of the analyte. Figure 3 presents an example of this approach in which a specific tryptic peptide of the coagulation protease prothrombin is measured in a tryptic digest of plasma. This measurement, based on precise molecular characteristics of the peptide, is in fact more specific for prothrombin than a typical immunoassay (in which lack of perfect antibody specificity is usually overlooked). An internal standard is often spiked into the sample to provide a reference signal to which the analyte is compared for absolute quantification. Lower limits of quantification (LLOQ) of 5–25 ng ml<sup>-1</sup> (~20 nM) can be obtained for drug metabolites (Zhang *et al.* 2000a), and < 10 ng ml<sup>-1</sup> for pesticides in vegetable samples (Sannino, 2004). In serum and plasma, methods based on two-stage mass spectrometry (MS/MS) quantify the drugs mycophenolic acid (Streit *et al.* 2004) (0.5 ng ml<sup>-1</sup>) and sirolimus (Streit *et al.* 2002) (0.25 ng ml<sup>-1</sup>), as well as hormones and metabolites such as thyroid hormone T3 (Tai *et al.* 2004) (a reference method with coefficient of variation (CV) < 3%), homocysteine (Magera *et al.* 1999; Arndt *et al.* 2004; Stabler & Allen, 2004), S-adenosylmethionine and S-adenosylhomocysteine (Struys *et al.* 2000) (LLOQs of 3 and 1 ng ml<sup>-1</sup>, respectively, with CV < 8%).

The SPE–LC–MS/MS approach (where LC is liquid chromatography) has also been successfully applied to peptides, which typically have higher masses than the small molecules discussed above. Peptides yield specific fragments suitable for MS/MS measurement, and suitable internal standard peptides can be prepared by chemical synthesis. Small amounts (picomoles) of neuropeptides (enkephalins (Desiderio & Kai, 1983), endorphins (Dass *et al.* 1989), substance P (Lisek *et al.* 1989)) were detected by MS/MS and measured against stable isotope-labelled standards in the 1980s. More recently this approach has been used in standardized assays for larger peptides in serum such as 3 kDa thymosin a1 (LLOQ 0.5 ng ml<sup>-1</sup> (Tuthill *et al.* 2000) CV < 10%) and for small proteins like the 10 kDa recombinant protein rK5 (LOQ 100 ng ml<sup>-1</sup> in monkey serum (Ji *et al.* 2003) and later 10 ng ml<sup>-1</sup> in human serum (Ji *et al.* 2004), CV of 3%). The structural specificity of MS/MS allows better analyte discrimination than immunoassays: particular forms of insulin and its fragments can be selectively detected (Kippen *et al.* 1997), and in fact MS/MS is now used as a reference against which to standardize different immunoassays for C-peptide (Fierens *et al.* 2003).

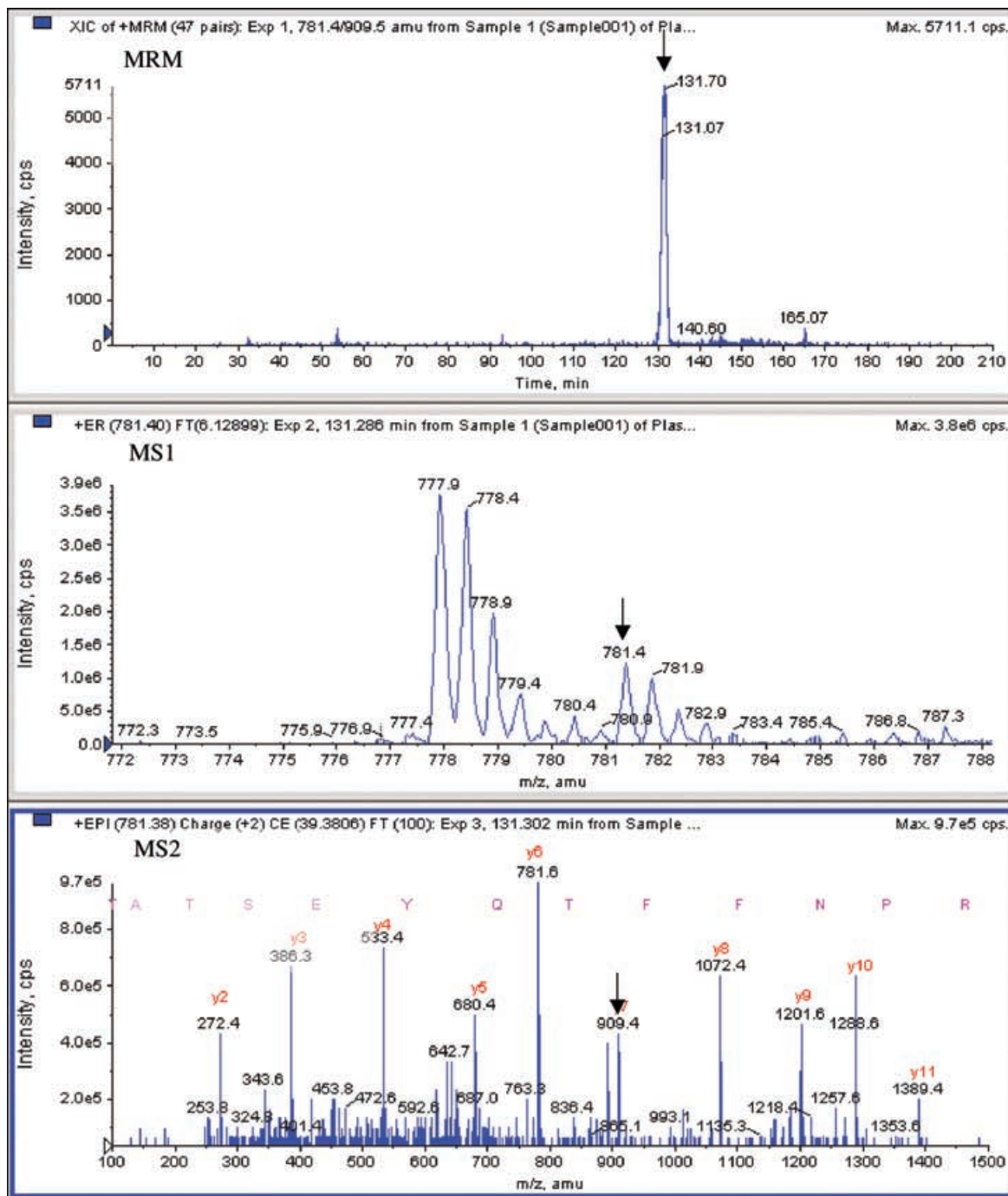
However, the method as described above is not generally useful for proteins larger than about 10 kDa, whose higher mass is not as well resolved by current MS or LC systems as peptides, which do not fragment efficiently into a few discrete pieces, and for which labelled

internal standards are significantly more expensive. MS analysis of whole proteins from plasma is typically restricted to non-quantitative applications in which an available high affinity antibody is used to capture the protein, which is then eluted and analysed by MS (Kiernan *et al.* 2003; Nepomuceno *et al.* 2004), or digested to peptides that can be subjected to MS/MS for structural analysis (Labugger *et al.* 2003; Nedelkov *et al.* 2004). Such methods are useful for detecting protein sequence variants and post-translational modifications (PTMs), and can be quantitative in the rare cases where a purified cross-reacting homologue from another species is available to serve as an internal standard (e.g. the assay of 7.6 kDa IGF1 (Nelson *et al.* 2004) at ~100 pg ml<sup>-1</sup>).

Thus in order to effectively leverage the successful methods of LC–MS/MS quantification to proteins in a sample such as plasma, one must ‘disassemble’ each protein quantitatively into its constituent peptides by complete chemical or enzymatic cleavage. Within this digest one can select a *monitor peptide* to serve as a quantitative surrogate for the protein, and achieve accurate quantification by spiking with a stable isotope-labelled version of the same peptide as internal standard (Stemann *et al.* 2001). Such ‘postdigest’ assays have been generated for some higher-abundance plasma proteins such as ApoA-I lipoprotein (Barr *et al.* 1996) (CV < 4%) and Hb A1C (Jeppsson *et al.* 2002) (an International Federation of Clinical Chemistry reference method in which a glycosylated peptide is measured with interlaboratory CVs of 1.4–2.3%). Attempts to assay the 26 kDa cancer marker prostate-specific antigen (PSA) (Barnidge *et al.* 2004) using a standard LC–MS/MS system yielded a detection limit of 4.5 mg ml<sup>-1</sup> (0.17 mg ml<sup>-1</sup> of the monitored peptide, a level ~1000 times higher than the clinically relevant level), while measurement of CRP (after a molecular weight enrichment by SDS gel) yielded quantitative measurements at < 1 mg ml<sup>-1</sup> (Kuhn *et al.* 2004).

While individual analytes within each class of molecule vary, the published data lead us to conclude that serum concentrations in the order of 1 ng ml<sup>-1</sup> for drugs, 1–10 ng ml<sup>-1</sup> for plasma peptides, and ~100 ng ml<sup>-1</sup> for peptides in a complex plasma digest can be measured by existing LC–MS/MS-based assay methods. On average, proteins in plasma are ~34 times as large as the roughly 10 amino acid-long monitor peptides chosen to represent them, and thus the protein detection limit (measuring a peptide in a digest) would be expected to be roughly 3 mg ml<sup>-1</sup>.

Two additional elements are required to enable quantitative MS/MS for targeted proteomics: the capability to assay many proteins at a time and a means to extend sensitivity downwards to the level of low abundance biomarkers such as cytokines (~10 pg ml<sup>-1</sup>).



**Figure 3**

An example showing MS/MS detection of a prothrombin peptide (TATSEYQTFNPR) in a tryptic digest of unfractionated plasma, using the SRM transition 781.4/909.7 (parent/fragment masses). Prothrombin is present in normal plasma at  $100 \text{ mg ml}^{-1}$ , and the peptide is detected at a signal-to-noise ratio (S/N, smoothed peak height/3 s background) of 85. In the figure, the arrow in panel MS1 shows the peak in the peptide MS spectrum selected as the parent, the arrow in panel MS2 shows the fragment chosen from the MS/MS spectrum (the y6 ion), and panel MRM shows the ion current detected at this parent/fragment SRM transition (with unit mass windows) over the entire course of a 3 h LC run. The MS/MS spectrum in MS2 unambiguously identifies the prothrombin peptide by sequence, providing absolute specificity better than immunoassay.

Multi-analyte methods are implemented in TQMS by rapidly switching between pairs of MS/MS parameters during the LC run. Published methods have measured up to 29 pesticides in one run (Barr *et al.* 2002) and prototype studies of up to 200 multiple-reaction monitoring (MRM) analytes performed. Sensitivity of MS assays can be increased by additional stages of fractionation prior to LC-MS/MS. Two such methods of particular promise involve the subtraction of specific high-abundance plasma proteins (e.g. albumin, transferrin, Igs, haptoglobin, etc.) using specific antibody columns (Pieper *et al.* 2003b), and the specific enrichment of selected monitor peptides through binding and release from anti-peptide antibody columns (Anderson *et al.* 2004a). The former method provides a 10-fold improvement in sensitivity (by subtracting 90% of the mass of protein in plasma), while the latter method yields an additional 100-fold average improvement using relatively crude rabbit polyclonal antibodies. These extensions provide a reasonable basis for the expectation that panels of 20–50 protein analytes taken from the top 6 or 7 (of 10) orders of magnitude plasma concentration should be accessible for routine MS/MS measurement.

### Candidate markers of cardiovascular disease

Given a technology platform for measuring a limited number of identified proteins, intelligent candidate selection is a high priority. As an example of a set of candidates to start with, I present here a table of proteins reported to have some connection with cardiovascular disease (here considered in a broad sense, and including heart disease, stroke, vascular disease, hyper- and hypo-coagulation) from literature and other sources (Table 1).

Cardiovascular disease (CVD) is the leading cause of death in the United States (~40% of all deaths), and a major economic burden (\$227 billion in direct medical costs this year) (2003). In 2001, there were more than 4 million visits to emergency departments with a primary diagnosis of CVD, and more than 6 million inpatient cardiovascular operations and procedures were performed (American Heart Association, 2003).

Cardiovascular disease includes a range of phenomena differing markedly in timescale, physical size, and relative effects of genes and environment. It includes slow processes such as atherosclerosis, which can evolve over decades, and very rapid events such as myocardial infarction, which can be lethal in a matter of minutes. It involves subtle changes at the molecular level, as coagulation enzymes are activated at the site of a ruptured arterial plaque, and large-scale physical consequences, when a blood clot physically plugs a major coronary artery. Genetic factors (e.g. familial hypercholesterolaemia or levels of lipoprotein (a) (Lp(a)) are strongly involved, as are environmental

and lifestyle factors, the most obvious of which are lipid intake and smoking. Largely on account of this breadth of causes and effects, and the diversity of treatment strategies that this makes possible, major progress has been made in the development of life-saving interventions. Damaged hearts can be repaired physically, by coronary artery bypass grafting (CABG) or percutaneous coronary intervention (PCI), or enzymatically, by administering recombinant human tissue plasminogen activator (tPA) to digest a clot; elevated blood pressure can be controlled by several different classes of drugs, and coagulation can be enhanced (in treatment of haemophilia by replacement of missing clotting factor proteins) or diminished (with aspirin, heparins, and platelet GP IIb/IIIa receptor antagonists).

A major challenge in medicine is thus deciding when, and upon whom, these effective interventions should be carried out. A patient presenting with chest pain may have an acute myocardial infarction (MI) requiring immediate PCI or tPA treatment, stable angina requiring nitroglycerine, oesophageal spasm with no cardiovascular consequences, etc. Given the urgency of this issue, the cardiology community has promulgated detailed guidelines concerning triage of chest-pain patients (Ryan *et al.* 1996; Braunwald *et al.* 2000). Perhaps most importantly, there is a window of opportunity, while conditions such as atherosclerosis and hypertension gradually worsen, in which the ability to anticipate an imminent acute event (e.g. MI or stroke) can have immense benefit. Where causal molecules or telltale molecular fingerprints can be identified, objective and reproducible laboratory tests can be created, helping to implement best medical practices at institutions large and small. Such tests are typically inexpensive in relation to drug treatment or surgical intervention, providing a major health economic benefit. And they can be fast, providing critical results in < 15 min when implemented in automated instruments near the patient.

**History of protein markers in CVD.** Cardiovascular disease is the most likely area in the spectrum of human disease to yield protein markers in plasma. Most pathologies of the cardiovascular system involve plasma proteins directly (e.g. the coagulation cascade with its positive and negative modulators (> 29 proteins), or proteins of lipid transport involved in atherosclerosis (> 16 proteins)), or proteins that interact with vessel walls, platelets, or both. In addition to these, numerous inflammatory modulators transported in the blood have direct and indirect relationships to cardiovascular disease, while release of proteins from the heart itself provides evidence of cardiac damage.

Consistent with this expectation, a number of very successful protein diagnostics have emerged in cardiovascular medicine. The most definitive of these is cardiac

**Table 1. A table of 177 candidate markers of cardiovascular disease (CVD) and stroke, assembled through literature search**

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
1	activin A	P08476	6.0E +02	(Eldar-Geva <i>et al.</i> 2001)	Released by heparin from vascular endothelium (Phillips <i>et al.</i> 2000)			
2	adiponectin (ADPN)	Q15848	4.8E +06	(Mallamaci <i>et al.</i> 2002)	Higher levels in essential hypertensives (Mallamaci <i>et al.</i> 2002)			
3	albumin	P02768	4.1E +10	(Specialty Laboratories, 2001)	Negative acute phase reactant, lower levels associated with increased risk of cardiovascular mortality (Shaper <i>et al.</i> 2004)			+
4	aldolase C	P09972	4.0E +03	(Asaka <i>et al.</i> 1990)	A more specific and sensitive marker of cerebrovascular diseases than aldolase A (Asaka <i>et al.</i> 1990)			
5	alpha 2 antiplasmin (alpha 2 AP)	P08697	7.0E +07	Progen test insert	An important regulator of the fibrinolytic system	+		
6	alpha 2 macroglobulin (alpha 2 m)	P01023	1.8E +09	(Specialty Laboratories, 2001)	Major plasma protease inhibitor			
7	alpha(1)- antichymotrypsin (ACT)	P01011	4.2E +07	(Putnam, 1975)	Major plasma protease inhibitor			+
8	alpha1 acid-glycoprotein (AAG)	P02763	6.9E +08	(Specialty Laboratories, 2001)	Acute phase reactant			+
9	alpha1-antitrypsin (AAT)	P01009	1.4E +09	(Specialty Laboratories, 2001)	Major plasma protease inhibitor			
10	angiotensin-converting enzyme (ACE)	P12821			Lower in stroke patients than controls (Catto <i>et al.</i> 1996)			
11	angiotensinogen	P01019	1.5E +06	(Bloem <i>et al.</i> 1995)	Precursor of major blood pressure control peptide			
12	antithrombin III (AT III)	P01008	2.0E +08	(Kalafatis <i>et al.</i> 1997)	Major inhibitor of thrombin	+		

(continued)



Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
13	apolipoprotein A-I	P02647	1.4E +09	(Glowinska <i>et al.</i> 2003)	Low level associated with mortality and myocardial infarction five years after CABG(Skinner <i>et al.</i> 1999)		+	+
14	apolipoprotein A-II	P02652	2.4E +08	(Luo & Liu, 1994)	Lipoprotein		+	
15	apolipoprotein A-IV	P06727	1.6E +08	(Kondo <i>et al.</i> 1989)	A relatively independent risk factor for CHD (Warner <i>et al.</i> 2001)		+	
16	apolipoprotein B	P04114	7.3E +08	(Glowinska <i>et al.</i> 2003)	Major component of LDL		+	
17	apolipoprotein C-I	P02654	6.1E +07	(Riesen & Sturzenegger, 1986)	Lipoprotein		+	
18	apolipoprotein C-II	P02655	3.3E +07	(Bury <i>et al.</i> 1986)	Lipoprotein		+	
19	apolipoprotein CIII	P02656	1.2E +08	(Onat <i>et al.</i> 2003)	Marker of CHD independent of cholesterol (Onat <i>et al.</i> 2003)		+	
20	apolipoprotein D	P05090			Lipoprotein		+	
21	apolipoprotein E	P02649	4.0E +07		Presence of epsilon4 allele a strong independent predictor of adverse events (Brcsic <i>et al.</i> 2000)		+	
22	apolipoprotein L1	O14791			Lipoprotein		+	
23	aspartate aminotransferase, mitochondrial (m-type)	P00505			Giagnostic for early detection of myocardial infarction (Yoneda <i>et al.</i> 1992)			
24	basic fibroblast growth factor (bFGF)	P09038	6.0E +03	(Song <i>et al.</i> 2002)	sICAM-1level increases in acute cerebral infarction (Song <i>et al.</i> 2002)			
25	beta(2)-glycoprotein I, nicked	P02749			May control extrinsic fibrinolysis via a negative feedback pathway loop (Yasuda <i>et al.</i> 2004)	+		

(continued)

Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
26	B-type neurotrophic growth factor (BNGF)	P01138	7.0E +02	(Reynolds <i>et al.</i> 2003)	Candidate stroke marker (Reynolds <i>et al.</i> 2003)			
27	cathepsin B	P07858	2.1E +03	(Kos <i>et al.</i> 1998)	Potential biomarker for vulnerable plaques (Chen <i>et al.</i> 2002)			
28	CD105 (endoglin)	P17813	3.4E +04	(Takahashi <i>et al.</i> 2001)	Potential myocardial infarction and stroke marker (Li <i>et al.</i> 1998)			
29	CD40 ligand, soluble (sCD40L)(= CD154)	P29965	2.9E +03	(Schonbeck <i>et al.</i> 2001)	Patients with unstable angina have elevated plasma levels of soluble CD40L (Schonbeck <i>et al.</i> 2001)			
30	ceruloplasmin	P00450	2.8E +08	(Kim <i>et al.</i> 2002)	Ceruloplasmin reported to be an independent risk factor for cardiovascular disease (Kim <i>et al.</i> 2002)			+
31	chitotriosidase	Q13231			Significantly increased in individuals suffering from atherosclerosis disease (Artieda <i>et al.</i> 2003)			
32	cholesteryl ester transfer protein (CETP)	P11597	1.9E +06	(Sasai <i>et al.</i> 1998)	Alleles affect CVD (Blankenberg <i>et al.</i> 2003)			+
33	chromogranin A	P10645	1.1E +05	(Ceconi <i>et al.</i> 2002)	Increased in chronic heart failure (Ceconi <i>et al.</i> 2002)			
34	clusterin	P10909	3.7E +08	(Hogasen <i>et al.</i> 1993)	Induced in media and neointima after vascular injury (Miyata <i>et al.</i> 2001)			+
35	coagulation Factor IX	P00740	5.1E +06	(Kalafatis <i>et al.</i> 1997)	Coagulation	+		
36	coagulation Factor V	P12259	6.6E +06	(Kalafatis <i>et al.</i> 1997)	Most common genetic CVD risk factor to date is a single point mutation (FV	+		

(continued)

Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
37	coagulation Factor VII	P08709	5.0E +05	(Kalafatis <i>et al.</i> 1997)	Leiden) (Dahlback, 2003) Coagulation	+		
38	coagulation Factor VII-activating protease	Q14520	7.5E +06	(Romisch <i>et al.</i> 1999)	Coagulation	+		
39	coagulation Factor VIII	P00451	2.0E +05	(Kalafatis <i>et al.</i> 1997)	Coagulation	+		
40	coagulation Factor X	P00742	1.0E +07	(Kalafatis <i>et al.</i> 1997)	Target for novel antithrombotic agents	+		
41	coagulation Factor XI	P03951	4.8E +06	(Kalafatis <i>et al.</i> 1997)	Coagulation	+		
42	coagulation Factor XII	P00748	3.0E +07	(Kalafatis <i>et al.</i> 1997)	Coagulation	+		
43	coagulation Factor XIIa	P00748	2.0E +03	(McLaren <i>et al.</i> 2002)	Levels of 2 ng ml <sup>-1</sup> or more have an increased risk of CHD (McLaren <i>et al.</i> 2002)	+		
44	coagulation Factor XIII	P00488, P05160	1.0E +07	(Katona <i>et al.</i> 2000)	Coagulation	+		
45	collagen I degradation byproduct (ICTP)	0			Altered in hypertrophic cardiomyopathy (Lombardi <i>et al.</i> 2003)			
46	collagen I synthesis byproduct (PICP)	0			Altered in hypertrophic cardiomyopathy (Lombardi <i>et al.</i> 2003)			
47	collagen I synthesis byproduct (PINP)	0			Altered in hypertrophic cardiomyopathy (Lombardi <i>et al.</i> 2003)			
48	collagen I synthesis byproduct (PIP)	0	1.0E +05	(Lopez <i>et al.</i> 2001)	May be useful to assess the cardioreparative properties of antihypertensive treatment in hypertensives (Lopez <i>et al.</i> 2001)			
49	collagen III propeptide (PIIIP)	0			(Nomura <i>et al.</i> 2003)			
50	collagen III synthesis byproduct (PIIINP)	0	5.0E +03	(Poulsen <i>et al.</i> 2000)	Correlates with infarct size in MI (Poulsen <i>et al.</i> 2000)			

(continued)

Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
51	complement C1 inactivator	P05155	3.0E +08	(Oshitani <i>et al.</i> 1988)	Can preserve ischaemic myocardium from reperfusion injury (Buerke <i>et al.</i> 1995)			
52	complement C3	P01024	1.3E +09	(Specialty Laboratories, 2001)	C3 is more strongly associated with previous myocardial infarction than other risk factors (Muscari <i>et al.</i> 2000)			+
53	complement C4	P01028	2.7E +08	(Specialty Laboratories, 2001)	Associated with previous myocardial infarction (Muscari <i>et al.</i> 1995)			+
54	C-reactive protein (CRP)	P02741	2.3E +06	(Menon <i>et al.</i> 2003)	CRP levels strongly predicts cardiovascular death (Park <i>et al.</i> 2002)			+
55	creatine kinase-MB	P12277, P06732			Specific biochemical marker of myocardial injury (Ay <i>et al.</i> 2002)			
56	endothelial cell protein C receptor (EPCR)	Q9UNN8	1.0E +05	(Kurosawa <i>et al.</i> 1997)	Protein C activation is augmented by EPCR (Esmon, 2003)			
57	endothelial leucocyte adhesion molecule 1 (ELAM-1)	P16581	9.2E +02	(Carson <i>et al.</i> 1993)	Stroke caused an initial transient increase of sELAM-1 (Fassbender <i>et al.</i> 1995)			
58	endothelin-1 (ET-1)	P05305	3.6E +00	(Tsutamoto <i>et al.</i> 1995)	ET-1 levels are elevated in acute MI (Monge, 1998)			
59	endothelin-1, Big	P05305	1.2E +01	(Erbas <i>et al.</i> 2000)	Elevated Big endothelin-1 is a strong predictor of atrial fibrillation (Masson <i>et al.</i> 2000)			

(continued)

Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
60	enolase, beta, skeletal muscle	P13929			Concentrations significantly increased in acute MI (Nomura <i>et al.</i> 1987)			
61	enolase, gamma, neurone-specific	P09104	9.6E +03	(Oh <i>et al.</i> 2002)	May be a useful marker for severity in acute ischaemic stroke (Oh <i>et al.</i> 2002)			
62	erythropoietin (EPO)	P01588	2.6E +02	(Masaki <i>et al.</i> 1992)	Protects neurones from hypoxic/ischaemic injury (Ehrenreich <i>et al.</i> 2002)			
63	E-selectin, soluble	P16581	1.5E +04	(Galvani <i>et al.</i> 2000)	sE-selectin significantly elevated in the acute stage of ischaemic stroke (Frijns <i>et al.</i> 1997)			
64	Fas, soluble (APO-1/CD95)	P25445	2.0E +03	(Ohtsuka <i>et al.</i> 1999)	Increased plasma sFas levels are predictive of future CVD (Trojanov <i>et al.</i> 2003)			
65	fatty acid-binding protein, heart-type (H-FABP)	P05413	2.0E +03	(Glatz <i>et al.</i> 1998)	Performs as well as myoglobin as a marker of cardiac reperfusion (de Groot <i>et al.</i> 2001)			
66	ferritin	P02792 + P02794	4.2E +04	(Zuyderhoudt <i>et al.</i> 1978)	Possible relationship with carotid atherosclerosis potentiated by LDL cholesterol (Wolff <i>et al.</i> 2004)			+
67	fibrinogen	P02671 + P02675 + P02679	2.5E +09	(Glowinska <i>et al.</i> 2003)	Strongly related to cardiovascular risk (Koenig, 2003)	+		+
68	fibrinopeptide A	P02671	9.0E +02	(Cronlund <i>et al.</i> 1976)	Increased in patients with			

(continued)

Table 1. Continued

Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
69	fibrinopeptide B beta 1–42	P02675			ACS and is associated with adverse outcome (Ottani & Galvani, 2001)		
70	fibrinopeptide B beta 15–42	P02675			May be predictive of recurrent ischaemia (Scharfstein <i>et al.</i> 1996)		
71	fibronectin	P02751	1.4E +06	(Castellanos <i>et al.</i> 2004)	Candidate haemostasis marker (Fareed <i>et al.</i> 1998)		
72	follistatin	P19883	6.0E +02	(Eldar-Geva <i>et al.</i> 2001)	Cellular fibronectin may be a marker protein for endothelial cell activation (Kanters <i>et al.</i> 2001)		
73	gamma-glutamyltransferase (GGT)	P19440			Released by heparin from vascular endothelium (Phillips <i>et al.</i> 2000)		
74	glial fibrillary acidic protein (GFAP)	P14136	4.5E +02	(van Geel <i>et al.</i> 2002)	Marker of liver dysfunction, alcohol intake and stroke (Whitfield, 2001)		
75	glycogen phosphorylase BB, cardiac	P11216	3.0E +03	(Hofmann <i>et al.</i> 1989)	Marker of brain damage (Herrmann <i>et al.</i> 2000)		
76	GMP-140 (soluble P-selectin)	P16109	2.0E +05	(Facer & Theodoridou, 1994)	Classical cardiac marker		
77	gp130, soluble (sgp130)	P40189	2.7E +05	(Li <i>et al.</i> 2001a)	Elevated in elderly hypertensives (Li <i>et al.</i> 2001b)		
78	GPIIb/IIIa, soluble	P08514			Correlated with variables reflecting deranged haemodynamic status (Aukrust <i>et al.</i> 1999)		
					Implicated in the pathogenesis of acute coronary syndromes (Wagner <i>et al.</i> 1998)		

(continued)

Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
79	growth hormone (GH)	P01241	2.0E +02	(Krassas <i>et al.</i> 2003)	Associated with an increased incidence of cardiovascular disease (Vahl <i>et al.</i> 1999)			
80	haptoglobin	P00737	6.2E +08	(Specialty Laboratories, 2001)	Subjects with Hp 2-2 had significantly higher serum total and free cholesterol concentration (Braeckman <i>et al.</i> 1999)			
81	haemopexin	P02790	7.6E +08	(Jakob, 2002)	Acute phase protein			
82	heparin cofactor II (HCII)	P05546			Protein inhibitor of coagulation (Mann <i>et al.</i> 2003)	+		
83	hepatocyte growth factor (HGF)	P14210	2.0E +02	(Matsumori <i>et al.</i> 2000)	Reflects the clinical course in patients with acute MI (Sato <i>et al.</i> 1997)			
84	hexosaminidase A	P06865			Subjects in the 95-100%ile showed significantly increased frequency of myocardial infarction of their fathers and of stroke in their mothers (Hultberg <i>et al.</i> 1994)			
85	hydroxybutyrate dehydrogenase (HBDH)	Q02338	1.3E +05	(Akenzua <i>et al.</i> 1992)	Mitochondrial enzyme useful for estimation of infarct size in MI (van der Laarse <i>et al.</i> 1984).			
86	immunoglobulin G	0	9.8E +09	(Specialty Laboratories, 2001)	Acute phase protein			
87	insulin	P01308	2.0E +03	(Green <i>et al.</i> 1976)	Serum insulin quantitatively associated with cardiovascular risk factors (Chen <i>et al.</i> 1999)			

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Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
88	insulin C-peptide	P01308	1.7E +03	(Donatelli <i>et al.</i> 1991)	C-peptide quantitatively associated with cardiovascular risk factors (Chen <i>et al.</i> 1999)			
89	insulin precursor (proinsulin)	P01308	4.3E +01	(Burtis & Ashwood, 1999)	Increased concentrations predict death and morbidity caused by CHD over a period of 27 years, independent of other major cardiovascular risk factors (Zethelius <i>et al.</i> 2002)			
90	insulin-like growth factor binding protein-1 (IGFBP-1)	P08833	6.0E +04	(Wacharasindhu <i>et al.</i> 2002)	Correlated negatively with several established cardiovascular factors (Heald <i>et al.</i> 2001)			
91	insulin-like growth factor-1 (IGF-1)	P01343	1.9E +05	(Oh <i>et al.</i> 2004)	May be a risk factor for certain cardiac disorders (Ren <i>et al.</i> 1999)			
92	intercellular adhesion molecule 1, soluble (sICAM-1)	P05362	5.3E +05	(Song <i>et al.</i> 2003)	sICAM-1 related to the estimated risk of coronary heart disease (Witte <i>et al.</i> 2003)			
93	interleukin-1 beta (IL-1 beta)	P01584	1.2E +00	(Lu <i>et al.</i> 2004)	Higher in MI group or UA (Wang <i>et al.</i> 2004)			
94	interleukin-1 receptor antagonist (IL-1Ra)	P18510			Plasma levels appear to be a valuable independent predictive factor of major adverse cardiac events in unselected patients undergoing PCI (Patti <i>et al.</i> 2002)			

(continued)



Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
95	interleukin-1 receptor family member, ST2	Q01638			Increased in the serum 1 day after myocardial infarction (Weinberg <i>et al.</i> 2002)			
96	interleukin-10 (IL-10)	P22301			Increased serum levels detected in stroke patients (Dziedzic <i>et al.</i> 2002)			
97	interleukin-18 (IL-18)	Q14116	5.9E +01	(Blankenberg <i>et al.</i> 2002)	Significantly increased in unstable angina and MI (Mallat <i>et al.</i> 2002)			
98	interleukin-2 (IL-2)	P60568	5.1E +01	(Mizia-Stec <i>et al.</i> 2003)	Significantly higher in patients with MI (Mizia-Stec <i>et al.</i> 2003)			
99	interleukin-6 (IL-6)	P05231			Increased serum level was a significant predictor of death or new heart failure episodes (Orus <i>et al.</i> 2000)			
100	interleukin-6 receptor, soluble (sIL-6R)	P08887	1.0E +05	(Disthabanchong <i>et al.</i> 2002)	Increased in MI and UA (Bossowska <i>et al.</i> 2003)			
101	interleukin-8 (IL-8)	P10145	1.7E +00	(Zhang <i>et al.</i> 2003)	Level higher in UA (Romuk <i>et al.</i> 2002)			
102	leptin	P41159			Patients with advanced CHF show elevated serum levels (Schulze <i>et al.</i> 2003)			
103	leptin receptor, soluble	P48357	2.3E +04	(Schulze <i>et al.</i> 2003)	Patients with advanced CHF show elevated serum levels (Schulze <i>et al.</i> 2003)			
104	lipoprotein lipase (LPL)	P06858	2.8E +05	(Dugi <i>et al.</i> 2002)	Significant association between the LPL protein mass and NYHA class		+	

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Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
105	lipoprotein receptor-related protein 1, soluble (sLRP1) (alpha-2-macroglobulin receptor)	Q07954	6.0E +06	(Quinn <i>et al.</i> 1997)	(Kastelein <i>et al.</i> 2000) May antagonize the clearance of ligands by cell bound LRP perturbing lipid metabolism (Quinn <i>et al.</i> 1997)		+	
106	lipoprotein(a) (Lp(a))	P08519	1.4E +08	(Glowinska <i>et al.</i> 2003)	An index of atherosclerosis risk (Malaguarnera <i>et al.</i> 1996)		+	
107	lipoprotein-associated phospholipase A2 (Lp-PLA2)	P04054	1.5E +03	(Kugiyama <i>et al.</i> 1999)	Potential biomarker of coronary heart disease, plays a proinflammatory role in the progression of atherosclerosis (Dada <i>et al.</i> 2002)		+	
108	L-selectin, soluble (sL-selectin) (CD62L)	P14151	6.7E +05	(Atalar <i>et al.</i> 2002)	CD62L expression increased during cardiopulmonary bypass (Hambusch <i>et al.</i> 2002)			
109	macrophage colony-stimulating factor (MCSF)	P09603	6.8E +02	(Saitoh <i>et al.</i> 2000)	Mean concentration in patients with coronary events was significantly higher than controls (Saitoh <i>et al.</i> 2000)			
110	matrix metalloproteinase-1 (MMP-1)	P03956			Patients with atrial fibrillation (AF) had lower levels of MMP-1 (Marin <i>et al.</i> 2003)			
111	matrix metalloproteinase-2 (MMP-2)	P08253	8.1E +05	(Noji <i>et al.</i> 2004)	Higher in hypertrophic cardiomyopathy than controls (Lombardi <i>et al.</i> 2003).			
112	matrix metalloproteinase-3 (MMP-3)	P08254	8.0E +03	(Sangiorgi <i>et al.</i> 2001)	Levels are strongly associated with carotid lesions			

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Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
113	matrix metalloproteinase-9 (MMP-9)	P14780	9.0E +03	(Sangiorgi <i>et al.</i> 2001)	(Beaudeau <i>et al.</i> 2003) Predicts haemorrhagic transformation in acute ischaemic stroke (Castellanos <i>et al.</i> 2003)			
114	monocyte chemoattractant protein-1 (MCP-1)	P13500	1.6E +02	(de Lemos <i>et al.</i> 2003)	Appears to play a crucial role at multiple stages of atherosclerosis (de Lemos <i>et al.</i> 2003)			
115	myelin basic protein (MBP)	P02686	2.5E +03		Marker of cerebral damage (Zhou <i>et al.</i> 1992)			
116	myeloperoxidase (MPO)	P05164			Predicts increased risk for subsequent cardiovascular events (Baldus <i>et al.</i> 2003)			
117	myoglobin, cardiac (Mb)	P02144	4.2E +04	(Burtis & Ashwood, 1999)	Cardiac muscle damage marker			
118	myosin heavy chain, cardiac	P13533, P12883			Cardiac muscle damage marker			
119	myosin light chain I, cardiac	P08590	1.0E +03	(Uji <i>et al.</i> 1991)	Cardiac muscle damage marker			
120	myosin light chain II, cardiac	P10916	2.0E +03	(Hirayama <i>et al.</i> 1990)	Cardiac muscle damage marker			
121	natriuretic peptide, atrial, C-terminal (C-ANP)	P01160			Diagnostic utility in detecting left ventricular dysfunction (Lee <i>et al.</i> 2002)			
122	natriuretic peptide, atrial (ANP)	P01160	5.6E +01	(Goto <i>et al.</i> 2002)	Diagnostic utility in detecting left ventricular dysfunction (Lee <i>et al.</i> 2002)			
123	natriuretic peptide, atrial, N-terminal (N-ANP)	P01160			Diagnostic utility in detecting left ventricular dysfunction (Lee <i>et al.</i> 2002)			
124	natriuretic peptide, atrial, propeptide (31–67)	P01160			Increased moderately with primary pulmonary hypertension			

(continued)

Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
125	natriuretic peptide, brain (BNP)	P16860	1.9E +02	(Goto <i>et al.</i> 2002)	(Goetze <i>et al.</i> 2004) Diagnostic utility in detecting left ventricular dysfunction (Lee <i>et al.</i> 2002)			
126	natriuretic peptide, brain, N-terminal (NT-BNP)	P16860			Diagnostic utility in detecting left ventricular dysfunction			
127	natriuretic peptide, brain, pro-form (proBNP)	P16860			40-fold increase in primary pulmonary hypertension (Goetze <i>et al.</i> 2004)			
128	neurone-specific enolase (NSE)	P09104	8.0E +01	(Oh <i>et al.</i> 2002)	Significantly elevated in patients with acute cerebral infarction (Oh <i>et al.</i> 2002)			
129	neutral endopeptidase 24.11 (NEP)	P08473	2.5E +02	(Zhang <i>et al.</i> 1994)	A target for ACE-inhibitor-like drugs			
130	neutrophil gelatinase-associated lipocalin (NGAL)	P80188	8.7E +04	(Elneihoum <i>et al.</i> 1997)	Levels higher in stroke (Falke <i>et al.</i> 2000)			
131	neutrophil protease-4 (NP4)	P24158	2.3E +04	(Elneihoum <i>et al.</i> 1997)	Levels higher in stroke (Elneihoum <i>et al.</i> 1996)			
132	osteoprotegerin (OPG)	O00300	2.3E +02	(Browner <i>et al.</i> 2001)	Serum levels associated with cardiovascular mortality, may be a marker for vascular calcification (Browner <i>et al.</i> 2001)			
133	paraoxonase (PON1, 2, 3)	(P27169, Q15165, Q15166)	5.9E +07	(Kujiraoka <i>et al.</i> 2000)	Plasma levels influence the risk of developing cardiovascular disease (Getz & Reardon, 2004).		+	
134	phosphoglycerate mutase (PGM) B-type	P18669			Novel marker for diagnosis of cerebral stroke and its severity			

(continued)

Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
135	plasminogen	P00747	1.0E +08	(Marchal <i>et al.</i> 1996)	(Hayashi & Matuo, 2001) Major enzyme of thrombolysis	+		
136	plasminogen activator inhibitor (PAI)-1-antigen	P05121	4.2E +04	(Glowinska <i>et al.</i> 2003)	High plasma levels reported in coronary artery disease and stroke (Diamantopoulos <i>et al.</i> 2003)	+		+
137	platelet endothelial cell adhesion molecule-1, soluble (sPECAM-1)	P16284	6.6E +03	(Zeisler <i>et al.</i> 2001)	Stroke patients displayed statistically significant higher levels of sPECAM-1 in sera (Zaremba & Losy, 2002)			
138	platelet factor 4	P02776	7.7E +03	(Cella <i>et al.</i> 1983)	Elevated in brain lacunar infarctions with long-lasting signs (Oishi <i>et al.</i> 1999)			
139	platelet-activating factor (PAF) acetylhydrolase	Q13093			Deficiency associated with stroke, myocardial infarction, brain haemorrhage, and non-familial cardiomyopathy (Tjoelker & Stafforini, 2000)			
140	platelet-derived growth factor (PDGF)	P04085 + P01127	1.7E +02	(Cimminiello <i>et al.</i> 1994)	Increased levels in chronic arterial obstructive disease (Cimminiello <i>et al.</i> 1994)			
141	pregnancy-associated plasma protein A (PAPP-A)	Q13219			Elevated in acute coronary syndromes (Bayes-Genis <i>et al.</i> 2001)			
142	prorenin	P00797	3.7E +01	(Sealey, 1991)	Involved in blood pressure regulation			
143	protein C	P04070	3.7E +06	(Yan & Dhainaut, 2001)	Major regulator of haemostasis	+		

(continued)

Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
144	protein C inhibitor (PCI)	P05154	5.3E +06	(Laurell <i>et al.</i> 1992)	(Yan & Dhainaut, 2001) Inhibitor of key component of natural anticoagulant pathway	+		
145	protein C, activated (APC)	P04070	2.0E +03	(Yan & Dhainaut, 2001)	Key component of natural anticoagulant pathway	+		
146	protein S	P07225	2.1E +07	(Kalafatis <i>et al.</i> 1997)	Deficiency of protein S constitutes a major risk factor of venous thrombosis (Dahlback, 2004)	+		
147	protein Z	P22891			In the context of juvenile stroke, high plasma levels may represent a prothrombotic condition (Lichy <i>et al.</i> 2004)	+		
148	prothrombin	P00734	1.0E +08	(Kalafatis <i>et al.</i> 1997)	Coagulation	+		
149	prothrombin fragment 1.2	P00734	1.2E +03	(McKenzie <i>et al.</i> 1999)	Stroke patients had higher values than controls (Soncini <i>et al.</i> 2000)	+		
150	P-selectin glycoprotein ligand-1 (PSGL-1)	Q14242			Serum levels decreased during CV surgery (Osmancik <i>et al.</i> 2002)			
151	P-selectin, soluble (GMP-140)	P16109	4.7E +04	(Carter <i>et al.</i> 2003)	Significantly elevated in the acute stage of ischaemic stroke (Frijns <i>et al.</i> 1997)			
152	resistin	Q9HD89	1.5E +04	(Fujinami <i>et al.</i> 2004)	Concentrations of adipocytokines such as resistin and adiponectin determine inflammation status of vasculature, and in turn the			

(continued)

Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
153	S-100beta	P04271			progress of Atherosclerosis (Kawanami <i>et al.</i> 2004) A promising early biochemical marker for cerebral injury following cardiac surgery (Farsak <i>et al.</i> 2003)			
154	serum amyloid A protein (SAA)	P02735			Classical inflammation marker (with CRP)			+
155	serum placenta growth factor	P49763			Associated with the occurrence of subsequent preeclampsia (Su <i>et al.</i> 2001)			
156	sex hormone-binding globulin (SHBG)	P04278			A biological marker for insulin resistance, which is linked to cardiovascular risk in African-American women (Sherif <i>et al.</i> 1998)			
157	smooth muscle myosin heavy chain	P35749			Intracoronary level may be a biochemical marker for the prediction of restenosis (Tsuchio <i>et al.</i> 2000)			
158	tau protein	P10636			Correlated with brain infarct volume and disability after 3 months (Bitsch <i>et al.</i> 2002)			
159	thrombin activatable fibrinolysis inhibitor (TAFI)	Q9P2Y6	3.5E +06	(Wada <i>et al.</i> 2002)	Indirectly affects clot stability (Mann <i>et al.</i> 2003)	+		
160	thrombomodulin, soluble (sTM)	P07204	4.5E +04	(Blann <i>et al.</i> 1997)	Strong, graded, inverse association with incident coronary heart	+		

(continued)

Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
161	thrombospondin-1	P07996	2.0E +05	(Hayden <i>et al.</i> 2000)	disease (Salomaa <i>et al.</i> 1999) Might function as an alternative substrate for thrombus formation (Jurk <i>et al.</i> 2003)	+		
162	tissue factor (TF)	P13726	2.8E +02	(Zemanova <i>et al.</i> 2003)	Good predictor of cardiac allograft vasculopathy (CAV) (Yen <i>et al.</i> 2002)	+		
163	tissue factor pathway inhibitor (TFPI)	P10646	2.3E +04	(Nomura <i>et al.</i> 2003)	Significantly higher in acute MI (He <i>et al.</i> 2002)	+		
164	tissue inhibitor of metalloproteinases-1 (TIMP-1)	P01033	9.5E +04	(Noji <i>et al.</i> 2001)	Significantly higher in HCM patients than in control subjects (Noji <i>et al.</i> 2004)			
165	tissue inhibitor of metalloproteinases-2 (TIMP-2)	P16035	3.4E +04	(Noji <i>et al.</i> 2004)	Significantly higher in patients with HCM accompanied by systolic dysfunction (Noji <i>et al.</i> 2004)			
166	tissue plasminogen activator (t-PA)	P00750	7.3E +03	(Glowinska <i>et al.</i> 2003)	Predicted coronary events during a very long-term follow-up (Niessner <i>et al.</i> 2003)	+		
167	transforming growth factor-beta (TGF-beta)	P01137	4.5E +03	(Shariat <i>et al.</i> 2001)	Concentrations decreased in patients with coronary artery disease (CAD) (Tashiro <i>et al.</i> 2002)			
168	tropomyosin 1 alpha chain	P09493	2.0E +03	(Cummins <i>et al.</i> 1981)	Elevated ~50-fold in MI (Cummins <i>et al.</i> 1981)			
169	troponin I, cardiac	P19429	1.0E +03	(Kini <i>et al.</i> 2004)	A clinical marker of cardiac muscle damage			
170	troponin T, cardiac	P45379	3.0E +00	(Xue <i>et al.</i> 2003)	A clinical marker of cardiac muscle damage			
171	tumour necrosis factor receptor I,	P19438	8.9E +02	(Weiss <i>et al.</i> 1996)	Significant independent			

(continued)



Table 1. Continued

	Name	Accession	Normal concentration (pg ml <sup>-1</sup> )	Source for concentration	Reason	Coagulation	Lipo-protein	Acute Phase
	soluble (sTNF-RI)				predictor of cardiovascular mortality (Falke <i>et al.</i> 2000)			
172	tumour necrosis factor receptor II, soluble (sTNF-RII)	P20333	1.7E +03	(Weiss <i>et al.</i> 1996)	Increased in patients with CHF (Nowak <i>et al.</i> 2002)			
173	tumour necrosis factor-alpha (TNF-alpha)	P01375	8.3E +00	(Mizia-Stec <i>et al.</i> 2003)	Levels were elevated in all CAD groups (Mizia-Stec <i>et al.</i> 2003)			
174	vascular endothelial growth factor (VEGF)	P15692	3.2E +01	(Lavie <i>et al.</i> 2002)	Levels increased in patients with peripheral artery disease (PAD) (Makin <i>et al.</i> 2003)			
175	vitronectin	P04004	2.6E +08	(Hogasen <i>et al.</i> 1993)	A cofactor for rapid inhibition of activated protein C by plasminogen activator inhibitor-1 (Gechtman & Shaltiel, 1997)			
176	von Willebrand Factor (vWF)	P04275			Elevated plasma concentrations are increasingly recognized as a cardiovascular risk factor (Vischer <i>et al.</i> 1997)	+		
177	von Willebrand Factor, propeptide (vWf:AgII)	P04275	7.0E +05	(Vischer <i>et al.</i> 1997)	Could provide a sensitive plasma marker of acute endothelial secretion (Vischer <i>et al.</i> 1997)	+		

The common name, Swissprot sequence accession number, normal plasma concentration (and source of concentration measurement), justification for inclusion, and membership in one of three general CVD-related groups (coagulation pathway, lipid transport, and acute phase reactants) are tabulated. Concentrations are mean values where given, or a geometric average of high and low normal values where a range was given. Blanks occur where the search has not yet found reliable published values. Some entries have multiple accessions (multiple subunits separated by +, or lack of sufficient information to select among homologues separated by commas), and in some cases multiple candidates share a single accession (when different processed forms of one protein are considered separately).

troponin I (TnI, or the alternative TnT, both muscle contractile proteins) as a primary indicator of myocardial infarction (Jaffe, 2001), often in combination with the cardiac isozyme of creatine kinase (CK-MB) and

myoglobin. In this case, the diagnosis of MI typically includes a finding of elevated cardiac marker (e.g. TnI > 1 ng ml<sup>-1</sup>), leading to initiation of reperfusion treatment based on the knowledge that the marker signals

destruction of cardiac muscle tissue surrounding an infarct. Brain-type natriuretic peptide (Maeda *et al.* 1998) (BNP or NTproBNP), a molecule produced in and released by the left ventricle, has recently been adopted as an effective test for congestive heart failure. Because of the clinical importance of these tests, they are performed in very large numbers: ~85 million troponin assays and ~10 million BNP assays are performed each year. Similarly the levels of inflammation markers like C-reactive protein (Ridker *et al.* 1998) (CRP), lipoprotein(a) (Agewall & Fagerberg, 2002), fibrinogen (Kannel *et al.* 1992), and the apportionment of cholesterol between high- and low-density lipoproteins (Luria *et al.* 1991) (usually distinguished in assays by their protein components) all serve as valuable measures of cardiovascular risk.

In fact, *many* proteins in plasma show changes associated with cardiovascular disease states. Thus the strategy of seeking single-protein tests (each with a defined reference interval, or normal range, outside of which a patient value is clearly diseased) has been vigorously pursued. Unfortunately, in most cases these changes are not sufficiently specific to provide a test of useful predictive value: the change may be real but too small in relation to genetic and environmental 'noise', or it occurs with other diseases as well. Where useful biomarkers have emerged, the discovery and development of each test was the result of efforts over a number of years. The appearance of cardiac troponin in plasma in MI was reported in 1987 (Cummins *et al.* 1987), the test was introduced commercially in 1995, and it emerged as the core parameter for MI diagnosis in 2000 (Alpert *et al.* 2000; Braunwald *et al.* 2000). BNP, probably the most rapidly adopted new diagnostic test in CVD, was shown to be diagnostic for congestive heart failure (CHF) in 1996 (Yamamoto *et al.* 1996) and introduced as a commercial test in 2002. However, most markers have been under investigation for many years: myoglobin since 1977 (Rosano *et al.* 1977), cardiac fatty acid-binding protein (FABP) since 1992 (Kleine *et al.* 1992) and cardiac myosin light chain 1 since 1994 (Uchino *et al.* 1994). On average, there appears to be a delay of approximately 10 years between discovery of a CVD marker and its commercial implementation in a form that can benefit clinical medicine (assuming it is specific and sensitive). Reducing this time lag while maintaining the rigor of clinical validation is a high priority.

**Collection of candidate CVD markers.** Table 1 presents a set of proteins that are confirmed or potential plasma markers of some aspect of cardiovascular disease (in the heart, vessels or brain). To my knowledge, no comparable list of proteins associated with a specific disease area has been assembled and published. Results from several sources were pooled to generate this list. A large set (> 2000) of papers was selected through keyword searches on cardiovascular disease and stroke, and these were

classified and clustered using the RefViz program where titles and abstracts were scanned for protein names. A table of these proteins was constructed in an Excel spreadsheet, to which was added additional 'pathway'-derived potential markers derived from a literature survey of the protein components of coagulation and thrombolysis pathways, as well as acute phase reactants and known inflammatory markers. The resulting list comprised 177 protein targets, some of which were composed of multiple subunits, and some of which were different fragments of a single protein. Where possible, the normal plasma concentration was extracted from the literature references, or, in the case of existing clinical markers, from the normal range values used in test interpretation. These values are of critical importance in developing strategies for measurement: the 50 most abundant candidates are likely to be measurable by MS/MS (as in Fig. 3) without additional enrichment steps, while the others may require more elaborate sample preparation or fractionation prior to quantification.

While almost all of these candidates have been evaluated in some form of CVD or stroke, none has been surveyed across all forms of these diseases, and very few have been investigated jointly in the same sample sets. Thus these candidates include many proteins that have disease relationships that are significant (though not definitive enough to provide a specific single protein test): precisely the kinds of candidates from which multiplex panels of great specificity might be drawn.

Table 2 presents 28 additional known or candidate biomarkers of CVD that are not individual proteins. These include specific protein complexes, protein modifications, antibodies against specific proteins and smaller molecules (typically metabolites). While these markers are not directly accessible to the MS-based approach outlined here, they can be measured by immunoassay or by alternative MS-based methodologies.

## Discussion

This paper makes an argument for a candidate-based approach to protein biomarker development, supplementing the methods of classical proteomics that seek a complete analysis of a target proteome. Specific features of the plasma proteome, including its complexity and dynamic range, make it resistant to complete analysis in the near future. A targeted proteomics approach, aimed at selected candidates, can provide greater sensitivity and thus greater coverage of markers across the 10 orders of magnitude spanning known markers.

The fact that a non-exhaustive search for candidates related to CVD and stroke produced 177 different proteins (and protein forms) is revealing. A great deal of exploratory work has already been done, providing a targeted approach with an excellent starting point. The fact that most of these proteins have not yet become stand-alone clinical markers does not prevent them from providing incremental

**Table 2. Other candidate CVD markers**

Protein complexes	fibrinogen D-dimer	(Ince <i>et al.</i> 1999)
	plasmin-alpha(2)-antiplasmin complex (PAP)	(Sakkinen <i>et al.</i> 1999)
	thrombin-antithrombin III complex (TAT)	(Brodin <i>et al.</i> 2004)
	tissue factor pathway inhibitor-factor Xa (TFPI-Xa) complex	(Ohkura <i>et al.</i> 1999)
	tissue plasminogen activator (tPA)-plasminogen activator inhibitor-1 (PAI-1) complex (tPA/PAI-1 complex)	(Johansson <i>et al.</i> 2000)
Protein modifications	haemoglobin, glycosylated (HbA1c)	(Schillinger <i>et al.</i> 2003)
	lipoprotein(a), glycosylated	(Zhang <i>et al.</i> 2000b)
Antibodies to:	angiotensin II receptor (AT1)	(Fu <i>et al.</i> 2000)
	beta 2-glycoprotein I (beta2-GPI)	(Ebeling <i>et al.</i> 2003)
	cardiac actin	(Dangas <i>et al.</i> 2000)
	cardiac myosin	(Ebeling <i>et al.</i> 2003)
	cardiolipin (aCL)	(Dangas <i>et al.</i> 2000)
	chlamydial LPS	(Lowe, 2001)
	heat shock protein 65	(Birnie <i>et al.</i> 1998)
	oxidized LDL	(Ogawa <i>et al.</i> 2001)
	phospholipid [lupus anticoagulant (LA)]	(Guerin <i>et al.</i> 1998)
	prothrombin	(Guerin <i>et al.</i> 1998)
Smaller molecules	asymmetric dimethylarginine (ADMA)	(Tarnow <i>et al.</i> 2004)
	dehydroepiandrosterone sulphate (DHEAS)	(Jansson <i>et al.</i> 1998)
	folate	(Riddell <i>et al.</i> 2000)
	homocysteine (HCY)	(Abbate <i>et al.</i> 2003)
	kallidin (a tissue kinin)	(Wagner <i>et al.</i> 2002)
	malonyldialdehyde (MDA)	(Belboul <i>et al.</i> 2001)
	marinobufagenin (MBG)	(Fridman <i>et al.</i> 2002)
	melatonin	(Grote, 2004)
	<i>N</i> -acetyl-aspartate	(Stevens <i>et al.</i> 1999)
	oxidized phosphatidylcholine (OxPC, formed in OxLDL)	(Itabe, 2002)
uric acid	(Leyva <i>et al.</i> 1998)	

Twenty-eight candidate markers of other types relevant to cardiovascular disease and stroke. These occur in four categories: protein complexes (where the amount of protein in heteromultimer complexes provides separate information from the concentrations of individual components); protein modifications (where the amount of specifically modified protein is relevant); antibodies (where the corresponding antigen is specified); and smaller molecules (which are not proteins, but rather metabolites). The first three categories are ultimately accessible to modified proteomics approaches. A citation is provided for each, illustrative of the connection to cardiovascular disease or stroke.

statistical improvement to multiprotein panels yielding improved specificity.

Two other factors also motivate a targeted approach. In the limiting case, the number of human genes is relatively small (~25 000), and it might be reasonable to design specific MS-based assays (and ultimately antibodies for immunoassays) for all of these. Quantifying a major form of each human protein as a candidate disease marker is an attractive goal, though obviously far less comprehensive than the complete analysis goal (all forms of all proteins) implicit in the aims commonly expressed in proteomics.

A second and more practical factor favouring targeted assays is quantification itself. Most of the methods currently employed in proteomics can detect many proteins, but generally with poor quantitative accuracy. In particular when aiming for greatest sensitivity, proteome surveys of plasma detect quite variable subsets of proteins, even in repeat runs on the same sample. This makes it very

difficult to assemble a coherent analytical dataset, since proteins are typically detected in one run but not the next: the dataset is filled with holes. This is acceptable when one is looking for hints as to the involvement of individual proteins in specific processes, but it is a major disadvantage when trying to develop a statistical case associating a protein with a disease in the human population. In this case accurate determinations of a protein in each sample are needed, as one obtains from specific assays.

By fusing the approaches taken by proteomics, analytical chemistry and clinical chemistry, hybrid methods should emerge capable of rapidly expanding the range of biomarkers for the study of disease, ageing and physiology.

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