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# Proteomics: New technologies and clinical applications

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ABSTRACT

During the past decade, various genomics-based techniques have been applied with increasing success to the molecular characterisation of breast tumours, which has resulted in a more detailed classification scheme and has produced clinical diagnostic tests, which have been applied to both the prognosis and the prediction of outcome to treatment. Application of proteomics-based techniques is also seen as crucial if we are to develop a systems biology approach to the discovery of biomarkers of early diagnosis, prognosis and prediction of outcome to breast cancer therapies. However, proteomics is met with greater challenges to overcome that include optimising specimen handling and preparation, as well as determining the most appropriate proteomic platforms to apply to the identification of differentially expressed biomarker candidates and their subsequent validation. In this review, we explore some of the issues involved in specimen sampling for biomarker screening, proteomic methodologies used to identify biomarkers from clinical specimens including the isobaric tags for relative and absolute quantification (iTRAQ) system as well as strategies to validate biomarkers such as monitoring initiated detection and sequencing-multiple reaction monitoring (MIDAS-MRM). The ultimate goal is to be able to combine both genomics and proteomics-based approaches to the screening, discovery and validation of biomarkers of breast cancer that will help us move towards the individualisation and optimisation of treatment for patients.



## 1. Introduction

The past decade has seen a keen interest to take a systems approach towards discovering new biomarkers of breast cancer that allow the diagnosis and classification of patients with this disease.[1–5] The goal of these initiatives is to discover and validate the individual biomarkers, or groups of biomarkers called biosignatures, that allow the classification of cancers.[6,7] In studies where patient outcome data are known, these signatures can be associated and linked with treatment outcome.[8,9] After proper clinical validation, such biomarkers could serve to guide treatment of patients with similar or identical biomarker patterns.[8,10–14] Biomarkers can range from genomic changes, such as deletions, amplifications, polymorphisms and proteomic changes, such as differential protein expression or modification, or different metabolites that are differentially present in diseased and healthy tissues or patients.

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One of the remaining key challenges is how to discover proteomic differences between patients and healthy individuals, and which patient-derived specimen is better suited for diagnosis and staging. For example, a rapid screen diagnostic assay for early cancer detection should rely on plasma or serum,[4,15] while a staging and therapeutic decision-making process may be based on tumour biopsy material.[16,17] The key for early detection is to identify unique markers for neoplasia, and to develop ultra sensitive assays that have high specificity and sensitivity for their detection, and that function in high complexity samples, such as plasma.[18] Staging and theranostic biomarkers ideally differentiate between different tumour stages, or potential susceptibility to chemotherapeutic treatments.[19] Since tumours or at least biopsies are available, such markers could be discovered and eventually diagnosed directly from such materials. The challenge here is to employ reproducible and consistent sampling methods that are compatible with clinical and pathological practice.

## 2. Specimen sampling for biomarker screening

Sampling blood and its derived plasma or serum from a patient is among the least invasive techniques in medical practice.[4,5,20] It can be readily obtained in large quantities, and many diagnostic assays have been devised to quantify biomarkers in plasma or serum. While there are standardised phlebotomy isolation and plasma generation protocols used in every clinic, they are generally ill suited for biomarker discovery projects. Slight variations in phlebotomy protocol and timing from blood draw to plasma aliquoting and storage can result in significant changes due to proteolysis of plasma proteins by plasma born proteases[21] as well as due to other programmed changes in composition.[22,23] It is therefore critical, especially in cases when multi-centre studies are performed, to adhere to rigorous sampling protocols, and to conduct sample collection with phlebotomy tubes that incorporate a protease inhibitor cocktail to minimise proteolytic changes of plasma during blood draw, processing and subsequent storage.

Because blood and plasma circulate in the body, and plasma in addition circulates in intercellular spaces, it effectively samples most tissues. Conceivably, it can contain proteins, such as putative cancer biomarkers, albeit in low concentrations. It has been suggested that plasma contains over 10,000 protein species.[24] The dynamic range of concentrations spans 10 orders of magnitude, with 14 proteins so abundant that they make up around 95% of the total protein mass. This provides a unique analytical challenge, with most analytical instruments being only able to function across three orders of magnitude. Commonly used strategies to deal with the dynamic range issue include the immunodepletion of plasma with mixed antibody depletion columns, such as the top-12 and the top-14 IgY depletion columns marketed by Beckman and Genway, respectively.[25,26] The immunodepletion strategy has the advantage that the most abundant proteins are removed from plasma; however, this may also remove proteins, including precious biomarkers that are complexed with the abundant protein species such as serum albumin and immunoglobulin.

Immunodepletion is followed by multidimensional fractionation of proteins or tryptic peptides derived from these proteins.[4] Common steps include the fractionation with strong cation exchange columns, followed by reverse phase separation. Some successful biomarker screens have employed two-dimensional separation at the protein stage, while others have relied on the two or three-dimensional separation of peptide mixtures. In addition, surface-enhanced laser desorption/ionization-time-of-flight/mass spectrometry (SELDI-TOF/MS) employs a surface-based fractionation scheme for proteins, effectively separating protein mixtures based on their biophysical properties.[27] All these fractionation strategies serve the main purpose to reduce the complexity of protein samples, because the mass spectrometers are generally unable to decipher the identities of complex peptide or protein mixtures.

While methods for plasma separation have been well established and can be standardised, the same is not necessarily the case with excised tumour or needle biopsy specimens. Historically, established regimens led to rapid formalin fixation and paraffin embedding of specimens. The process of formalin fixation was initially thought to be incompatible with mass spectrometer-based biomarker identification strategies, resulting in a push for snap freezing tumour samples post surgery. Access to liquid nitrogen as well as delays in freezing the samples post surgery in a consistent manner has plagued this approach and has led to sample-handling induced variance, making the discovery of biomarker candidates more difficult. Several recent studies strongly suggest that the covalent protein-protein crosslinks of formalin-fixed, paraffin-embedded (FFPE) samples can be reversed under certain experimental conditions, enabling the subsequent digestion of tissues and their analysis by mass spectrometry methods.[28–31] Since standard operating procedures for FFPE samples are well established, and since FFPE tissue slices can be dissected by laser capture micro dissection devices, this method has the advantage to work with more consistent and standardised methods, resulting in lower variance due to sampling.[29,32–34] Another major advantage of this approach is that it enables the analysis and subsequent biomarker discovery of large historic sample collections where in many cases the therapeutic outcome is known. Preliminary data suggest that peptides derived from FFPE tissue are compatible with subsequent iTRAQ labelling or analysis by MIDAS-MRM-type quantitation approaches.

## 3. Identification of differentially expressed biomarker candidates

Methods to identify biomarkers from clinical specimens can be categorised into two principle methodologies: mass spectrometer-based methods and antibody array-based methods. In cases where the nature of the biomarkers or biosignatures is not known, mass spectrometry is most commonly employed to both map differences in detectable protein expression, as well as to identify the proteins that are differentially expressed.[35] Different groups have employed different mass spectrometer-based relative quantitations. Among the three most commonly employed methods are

SELDI-TOF, iTRAQ coupled to liquid chromatography (LC)–MS/MS,[36,37] and different variations of spectral abundance or total ion current counting of peptides.[38] SELDI-TOF uses different capture surfaces to separate proteins based on their biophysical properties.[39,40] TOF/MS is then used to determine protein profiles, which are analysed by software to detect and quantify differences in protein expression profiles. To identify differentially expressed proteins of interest, proteins have to be eluted from SELDI chip surfaces and analysed after trypsinisation, which adds to the time and cost of this approach.

Since most clinical samples that are to be compared have similar composition, identically prepared samples, after sufficient protein and peptide separation (to reduce sample complexity), can be directly compared in sequential mass spectrometer runs without the use of labels. Some methods compare the total ion current for a given peptide at a given point in time during the separation, while the others compare the number of times a unique MS/MS spectrum has been sampled. Assuming that the samples have similar complexity and that the mass spectrometers and separation columns have been well calibrated with established standards, this approach has been successfully used in major biomarker discovery projects.[38]

Another approach that has been successfully used in the discovery of biomarkers from a variety of clinical specimens is the iTRAQ approach[36,37,41–44] iTRAQ labels are isobaric labels that react with primary and secondary amines of peptides. Current marketed versions of iTRAQ labels contain four or eight labels as part of a labelling kit. Each label has an identical mass but fractionates differentially during collision-induced fragmentation while performing MS/MS analysis. For example, peptides labelled with the 114 reagent will generate a 114 *m*/z ion in an MS/MS spectrum, whose peak area is proportional to the relative abundance of the peptide-derived protein in the original biological sample. By mixing four or eight differentially labelled samples followed by further separation and MS/MS analysis, one can then determine the ratio between the different isobaric reporter ions, which indicate the fragmented peptide's relative abundance level in different samples.

To perform differential labelling of clinical samples with iTRAQ, individual samples are trypsinised and each trypsinised sample is labelled with a different isobaric label (e.g. 114, 115, 116 and 117). Differentially labelled peptide mixtures are then combined at equimolar concentrations and further separated by two-dimensional chromatography. As peptides enter the mass spectrometer, individual peptides are fragmented, and for a given peptide species the amino acid sequence is determined, as well as the ratio is determined between different iTRAQ reporter ions that represent a direct relative abundance comparison between the original samples. The advantage of the iTRAQ labelling method over other relative quantitation methods is that after trypsinisation of samples and iTRAQ labelling and mixing of samples, all subsequent separation conditions are identical, resulting in very low coefficients of variance and faster analysis. By including a pooled standard control against which all samples are compared, relative quantitation is possible across large patient cohorts. This approach has been successfully employed for the discovery of biomarkers from both plasma and tissue.[36,41,42,45–50]

Differences in protein expression levels alone are only one category of putative cancer biomarkers. In many instances, cancer is characterised by aberrations in signal transduction cascades that often manifest themselves in differentially activated signal transduction molecules,[51] which can lead to differential post-translational protein modifications, such as phosphorylation. While elegant mass spectrometry approaches exist to compare differentially expressed phosphopeptides, it is possible to test for key pathways through targeted antibody arrays, which appear to be more cost-effective.[52,53]

Much literature has been devoted to determining which biomarker discovery strategy is better, or which mass spectrometer-based method is superior. While well-controlled comparative studies have been rare, most studies, if well-controlled, have their own merit, as long as the analytical methods are standardised. In the recent years, a shift has been seen from SELDI-TOF discovery, to direct spectral or spectral abundance-based comparison, over to label-based methods. When assessing these technological advances, it is important to pay attention to the standard metrics that describe the accuracy and reproducibility of the method employed. For example, a routine iTRAQ experiment will have a coefficient of variance between 10% and 15%, which is significantly lower than variance introduced by sample preparation.[54]

Various bioinformatics packages developed by mass spectrometer vendors as well as academic initiatives can then be employed to ultimately provide information, such as the relative expression level of a protein identified by virtue of its individual peptides. Clustering algorithms, akin to the ones used in gene expression profiling, can determine patterns of expression among groups of biomarkers and/or groups of patients. A key aspect missing so far from biomarker analysis projects is a clear strategy to differentiate between fortuitous changes in protein expression levels, and bona fide biomarker candidates. One possibility is to establish a global database with common variable plasma or tissue proteins whose change is a likely consequence of patient-to-patient variability or environmentally induced changes, rather than an association with a specific disease.

## 4. Validation of biomarker candidates

Since biomarker discovery projects are resource intensive, many studies use relatively small cohorts of well-characterised patients for discovery, followed by extensive validation of fewer biomarker candidates using less expensive high-throughput methods, such as enzyme-linked immunosorbent assays (ELISAs).[55] Typically, a cohort of a few hundred patient specimens is included in studies that are then screened by ELISA (in case of plasma) or immunohistochemistry (IHC), in case of tumour biopsies.

Since most validation screens rely on the generation of antibodies against candidate biomarkers, the validation is usually time and cost-intensive. A recently established mass spectrometer-based method termed MIDAS-MRM allows the rapid and cost-effective absolute quantitation of biomarkers in plasma and tissue extracts[56,57] MIDAS-MRM, first implemented on Applied Biosystem's Q-TRAP 4000 mass

spectrometer, allows the multiplexed detection of numerous characteristic peptides, and by inclusion of isotopically labelled peptides also absolute quantification. The fact that this methodology does not rely on antibodies, and that generally biomarkers that have been discovered by mass spectrometry screens also generate peptides that are detectable by MRM-based approaches, it offers itself as an ideal method for biomarker candidate validation across a large patient cohort. Future developments, such as scheduled MRM assays and protein and peptide enrichment strategies, should allow performing MRM-based protein quantification in higher multiplicity and with higher sensitivity.

Validated biomarkers that are associated with disease or treatment response will then have to be converted into diagnostic assays and diagnostic panels. These can be in the form of ELISA, IHC or as immuno-PCR assays for the early detection of cancer antigens in plasma.

## 5. Outlook

The field of breast cancer diagnostics and prognostics is greatly aided by some emerging technologies that promise to augment the existing, but rather limited, protein and metabolite biomarker portfolio. The remaining challenge is to improve methods of sample collection and preparation, which could be accomplished by introducing robotics to carry out tasks, such as tissue digestion, protein trypsinisation, iTRAQ labelling and buffer exchanges. To contain costs of biomarker validation, cheaper antibody-dependent and independent techniques have to be developed. Some encouraging developments are low-cost alternatives to ELISA assays, such as the label-free detection of protein on porous silicon surfaces, single molecule detection of biomarkers via quantum-dots or immuno-PCR schemes and better methods to compare glycoproteomes.

## Conflict of interest statement

None declared.

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