Harnessing the modified proteome for increased diagnostic power

Relevant biomarkers are vitally important in personalised medicine, guiding patient matching to specific therapies for earlier, easier and more effective disease identification and treatment. Here we trace the expectations of 'omics-based healthcare interventions, from the increased understanding stemming from genomic and transcriptomic revolutions, through the lack of validated proteomic biomarkers, to the developing recognition of the potential of the modified proteome. Examining traditional methods and new advances in the field of proteomics that allow such investigations, we will discuss how they are currently being employed, with the potential to improve patient outcomes and reduce healthcare costs.

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Precision medicine holds the potential for the treatment for many diseases that have proven unresponsive to traditional therapies, such as cancer and autoimmune conditions. This strategy has seen much promise, with the field of precision medicine predicted to increase from a global value of $38.92 billion in 2015 to $88.64 billion by 2022[1]. Precision medicine relies on the measurement of specific, objectively-quantifiable biomarkers in patient samples to match treatments and individual patients according to their specific genetics or biochemistry. For each specific disease these biomarkers may be predictive, prognostic or both.

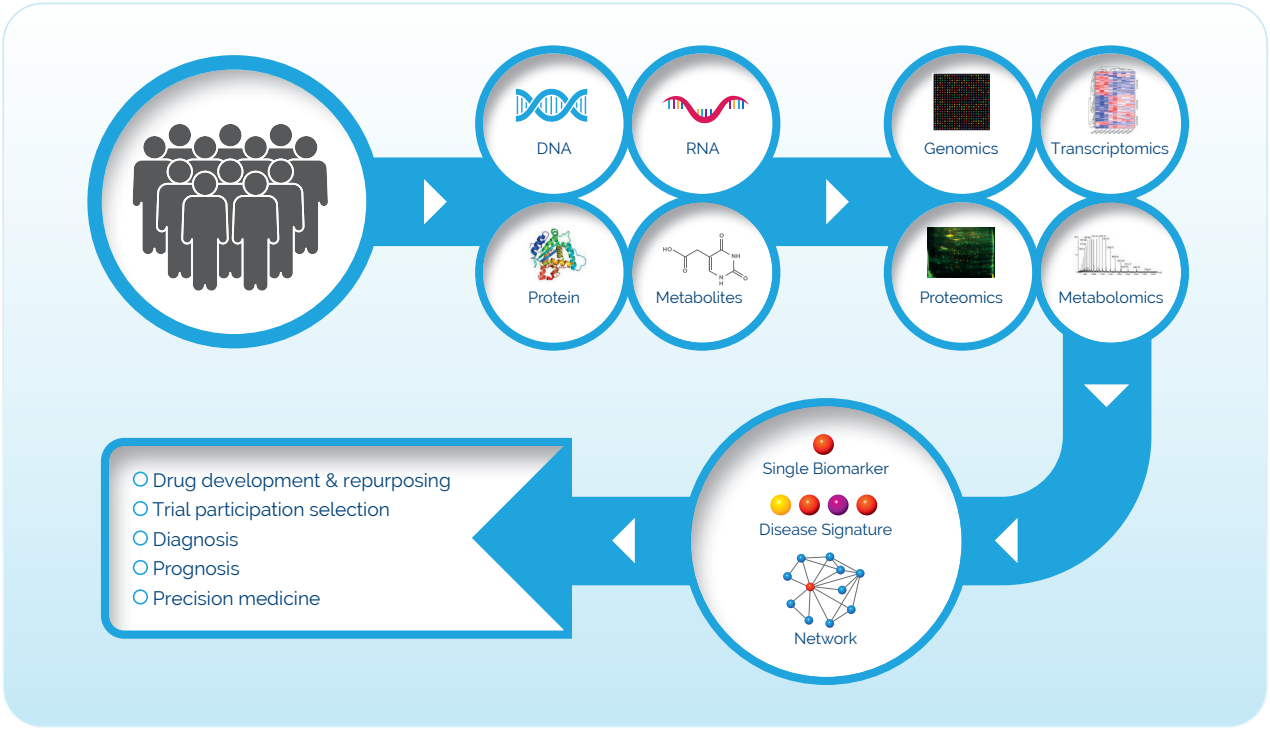
The aim of precision medicine is to improve the benefit-to-risk profile of many therapeutics by providing treatments only to patients who show compatible chemistry for a drug. In this way, non-compatible patients avoid the stress of undergoing unnecessary treatments and any potential toxic side-effects while also saving the high costs associated with such treatments. With costs for Merck's anti-PD-1 cancer immunotherapy blockbuster, Keytruda (Pembrolizumab), averaging at more than $100,000 per patient, and Novartis' CAR-T drug for leukaemia, Kymriah (Tisagenlecleucel), costing more than $350,000 per treatment, these cost savings are far from insignificant[2,3]. Indeed, the high cost of many precision medicines is a barrier to uptake for patients, healthcare services and payers across the globe.

Gleevec (Imatinib), a tyrosine kinase inhibitor manufactured by Novartis, is one example of a precision medicine therapeutic success story. Used in the treatment of chronic myeloid leukaemia, patients receiving Gleevec as a first-line treatment showed an overall 10-year survival rate of 83.3% compared to the 43-65% 10-year survival rate observed with previous treatments[4-6]. Herceptin (Trastuzumab) offers another milestone in the treatment of early and metastatic HER2 positive breast cancer, with 10-year survival rates showing improvement from 75.2% with chemotherapy alone to 84% with the use of Herceptin. Rates of survival without recurrence have also increased in response to Herceptin treatment from 62.2% to 73.7%[7]. Many other precision medicine drugs are

Figure 1

biosignatures

Omics technologies in precision medicine can help identify new disease biomarkers, for diagnosis, prognosis, patient stratification and drug development and repurposing.



showing promising trial results or have been licensed for clinical use by the FDA, often demonstrating potent and durable effects in patients. However, due to the targeted nature of these drugs and complex signalling networks at play in the tumour microenvironment, less than half of US patients with cancer were estimated to be eligible for precision medicine drugs in the form of cancer immune checkpoint inhibitors, with a projected response rate of only 12.46%[8].

Despite considerable investment in terms of time, money, human resource and logistics in precision medicine, this has failed to be accompanied by parallel increases in the number of diagnostic or therapeutic entities gaining regulatory approval. Questions regarding the value of this approach are being raised across the industry[9,10]. Reducing attrition rates and reversing the current productivity trend requires consideration of the approach to disease biomarkers, including the methods for stringent and reliable target selection and validation, along with the definition of reliable surrogate endpoints to facilitate effective clinical trials. If errors exist in the validity or measurement of biomarkers the whole framework surrounding precision medicine begins to crumble, thus precision medicine is effectively impossible without precision in the measurement and validation of biomarkers.

Precision medicine biomarkers in an omics landscape

Biomarkers are used in the precision medicine field within companion diagnostics to identify and stratify patients for treatment and to monitor therapeutic efficacy both during clinical trials and in post-launch follow-up studies. Beyond companion diagnostics, many are investigating the application of biomarkers in early diagnostic and screening programmes. Identification of diseases, notably various forms of cancer, at early and initial stages is associated with improved patient outcomes and significantly-reduced healthcare costs[11], hence identifying biomarkers that can diagnose disease early offers the potential to transform healthcare.

While many of the drug targets that have proven profitable in precision medicine were identified through molecular biological analysis of the specific disease physiology, this approach is slow,

arduous and associated with significant cost in the process of characterising 'gene-to-function'[12]. Increasing attrition in pharmaceutical pipelines and rising costs in bringing new drugs and diagnostics to market have resulted in a relentless focus on improving the speed and efficacy of target identification and development processes (eg, first to market) in the hope of attaining fast financial returns. Sophisticated large-scale methods are now available to profile diseases through genomics, transcriptomics, proteomics and metabolomics and identify biomarkers that define disease in terms of combined clinical, physiological and patho-biological abnormalities. The aspiration is that these approaches will bring more rapid and cost-effective results in biomarker discovery and validation, with a 'screen-to-gene' approach to improve diagnosis, facilitate the monitoring of disease and therapies and unravel underlying molecular pathways (**Figure 1**)[12].

Two of the most developed omics technologies are cancer genomics and transcriptomics. Tumour whole genome, whole exome and transcriptome sequencing are now feasible assays with high sample throughput, price tags in the order of $500-$1,500 per sample and comprehensive databases such as TCGA, COSMIC and ICGC being publicly available[13]. Genomics and transcriptomics have consequently become affordable and clinically feasible analytical techniques. This has led to a shift in the industry towards pharmacogenomics, which seeks to define genetic markers that predict individual responses to drugs to inform precision medicine. Competitors within this space are collaborating with governments and other agencies to enhance patient accessibility to diagnostic tests and products. Many genomic screening tests are now available to optimise disease treatment, particularly for cancer. These include Foundation Medicine's FDA-approved FoundationOne companion diagnostic screen for solid tumours. Performed on formalin-fixed paraffin embedded tumour tissue sections this Next Generation Sequencing (NGS)-based test offers a broad screen of microsatellite instability and tumour mutational burden, including BRCA1/2 analysis for ovarian cancer treatment with Lynparza® (olaparib) or Rubraca® (rucaparib); KRAS analysis for colorectal cancer treatment with Erbitux (cetuximab) and BRAF analysis for melanoma and non-small cell lung cancer treatment with Tafinlar® (dabrafenib); in addition to PD-L1 immunohistochemistry testing to inform therapy decisions[14].

Patient tissue biopsy is associated with limitations of cost, hospital time, patient trauma and inaccuracies of sampling from heterogenous tumour tissue. To overcome some of these issues there is an increasing interest in genomic analysis of liquid biopsy-based biomarkers. Liquid biopsy is a non-invasive method that combined with -omic approaches could be extremely useful in clinics to identify relevant biomarkers. In 2014, Guardant Health launched its NGS-based 70 gene screen for common cancer mutations, Guardant360, which has since become one of the most frequently-used liquid biopsy screens to direct cancer treatment within the US[15]. The ability to multiplex using genomic tests and target a plethora of different biomarkers within one screen increases the diagnostic capability, applicability and value of such assays. Guardant Health further launched GuardantOMNI in 2017, an NGS-based assay for use in patient stratification of clinical trials and monitoring response to therapeutics during clinical development of biopharmaceutical drugs and is developing additional screening assays for early cancer diagnosis and detection of cancer recurrence using this technology.

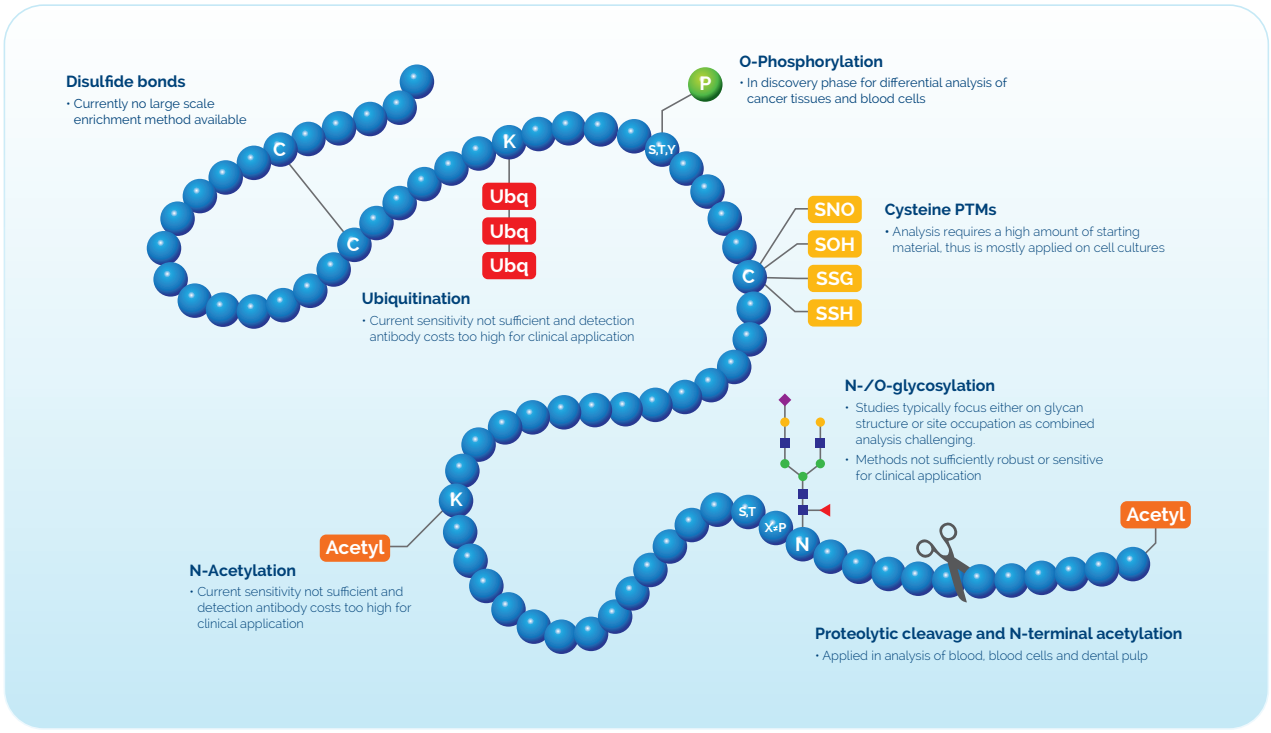
With investment of more than $1.5 billion since 2016[16], GRAIL is exploring genomic screening via NGS with the aim of identifying blood-based biomarkers for the early detection of cancer from circulating free DNA (cfDNA). It aims to generate an atlas of the circulating cell-free genome through analysis of more than 15,000 individuals with various cancer states to allow the simple and rapid early diagnosis of cancers based on cfDNA biomarkers[11,17]. Others pursuing genomic and transcriptomic routes to biomarker identification and clinical implementation using liquid biopsies are Freenome and Thrive, both of which are utilising genomic techniques alongside traditional protein analysis via immunoassay, and the Swiss-based Novigenix, which is investigating immune-based transcriptomic biomarkers for cancer diagnosis and prognosis and has launched a transcriptomic-based assay for the early detection of colorectal cancer from blood biopsies[18].

Despite considerable progress in large-scale proteomic methods, including improved detection limits and sensitivity, such methods have not yet been adopted into routine clinical practice. The main limitations that prevent integration into the clinic are the high cost of equipment, the need for highly-trained personnel and last, but not least, the establishment of reliable and accurate protein biomarkers or panels of protein biomarkers for disease detection. Compared to the genome and transcriptome, the proteome is far more complex and dynamic, being comprised of the basic protein

Figure 2

biosignatures

Common protein post-translational modifications and analytical methods.



sequences encoded within the genome, in addition to various isoforms and post-translationally modified variants[19]. Proteins are the functional molecules in the cell and as such represent actual physiological conditions. Consequently, their use as diagnostic biomarkers could be advantageous in detecting and monitoring pathological conditions by giving rise to earlier signals of disease or more accurate therapeutic monitoring. A myriad of protein biomarkers are already in use in clinical diagnostics, such as PSA in the detection of prostate cancer, HER2 in the detection of breast cancer[20,21] and PD-L1 in the selection of responsive patients to immunotherapy for lung cancer[22]. However, these markers were identified and validated prior to the omics via traditional molecular assays and their detection and analysis rely on lower throughput techniques such as enzyme-linked immunoassays (ELISAs) and other immunoassays[21].

For large scale proteomic measurements of proteins and peptides mass spectrometry (MS) is the current favoured method, yet at present this technique is more suited to protein biomarker research than clinical assay. The range of limitations of this technology include technical complexity of the methods, low throughput, high cost of assay, lack of thorough analytical and clinical validation of both the methods and the proteomic biomarkers identified using this technique and, finally, problems associated with the sensitivity of the testing for specific rare proteins in protein-rich clinical samples, such as plasma, serum, faeces or saliva[23]. Thus, despite offering potential for earlier and more accurate detection of disease and location of tumours in the body, large-scale screens for proteomic biomarkers are not currently at the same stage of development as their genomic counterparts.

Nonetheless, some companies have continued to pursue and refine this approach, typically coupled with liquid chromatography (LC-MS), for disease biomarker identification and analysis. In 2013, Integrated Diagnostics (Indi) brought to market a blood-based diagnostic assay, Xpresys Lung, that functioned on an MS platform. This assay determined the probability of lung nodules 8-30mm in size, that were identified via CT, being benign. Of

the approximately three million people in the United States who present with a lung nodule on CT each year, about 200,000 will have lung cancer; the rest will have benign lung nodules. A test result indicative of a high probability of being benign allowed patients to enter a period of watchful waiting without the need for more invasive diagnostic studies or biopsy. Indi was acquired by Biodesix in 2018[24]. More recently, German-based Uroquant is developing a diagnostic and prognostic assay for bladder cancer that relies upon quantitative MS analysis of biomarkers from urine samples[25].

Mining biomarkers in the modified proteome

Although techniques for the large-scale assay of proteomic biomarkers have largely failed to translate to the clinic and the proteomic biomarkers analysed via standard methodologies that have been integrated into healthcare practices arguably offer minimal diagnostic value[20], the promise of clinical proteomics may be realised given increasing evidence for the role of the modified proteome in disease.

Post-translational modification (PTM) of proteins offers a rapid, elegant and energetically efficient method to modulate and maximise the functionality of a single gene product, which may occur in a reversible manner. Throughout biology, PTMs are essential to regulate fundamental cellular processes. Every human protein has the potential to be post-translationally modified at least once during its life span and these cellular homeostatic switches represent critical and sensitive nodes for pathogenesis, both as drivers and markers. Aberrant PTMs have been associated with many diseases, ranging from the development of cancer to autoimmune disease. One identified reason underlying the failure of immuno-oncological strategies to deliver across large patient populations is due to the variation exhibited in individual patients' modified protein signatures[26,27]. Indeed, recent research has demonstrated that glycosylation, phosphorylation, ubiquitination, sumoylation and acetylation of PD-L1 all play a role in the stability of this tumour immune checkpoint ligand, with aberrant modifications modulating PD-L1 mediated immune resistance[28]. Consequently, each of these PTMs could act as novel, alternative biomarkers for diagnostics or as novel drug development targets.

Each protein sequence may have many different modifiable amino acids, but not all will be modified at the same time or within the same copy of the protein. Even for a specific residue competition may exist between different modifications, between acetylation and ubiquitination of lysine residues or between O-phosphorylation and O-GlcNac modification (**Figure 2**). Thus, each protein exists in an equilibrium of modified states, the balance of which can be shifted to drive different biological responses and it is often the state of the whole protein with all modifications included that is recognised by cell signalling systems. The known cancer driver p53 is an example of this whole protein PTM signature, where function and activity depend on the complete PTM pattern operating synergistically to modulate the protein structure, rather than individual PTMs[29-31]. This regulation via the whole protein signature is important for distinguishing modified protein biomarkers and in consideration of the methods used to detect them.

MS has been considered an unrivalled research tool for detecting and quantifying PTMs and their changes in a broad variety of samples. Consequently, more than 95% of current data on PTMs is derived from MS-based proteomic studies[32]. Methuselah Health is attempting to exploit MS analyses of PTMs to identify drug discovery biomarkers for the diseases of ageing, such as diabetes, neurodegeneration and autoimmunity[33]. However, analysing complete protein PTM patterns is a major challenge for MS. Current PTM research is restricted to the identification of peptides after proteolytic digestion (bottom-up proteomics), meaning information about the full PTM pattern across the protein is lost and often variation in PTMs are difficult to capture using this approach[34]. MS-analysis of the full PTM complement of the intact protein (top-down proteomics) is unfortunately unlikely to be applicable to clinical samples in the near future, as it is slow, expensive and limited to proteins <60kDa[35,36].

An alternative method utilised by Biosignatures to capitalise on the potential of PTM biomarkers within clinical samples is DIGE. This traditional technique offers sensitive analysis of whole protein PTM patterns[37,38], while offering the ability to multiplex assays for simultaneous detection of multiple biomarkers from a single patient sample. Using a next generation DIGE platform that delivers highly reproducible and accurate results[37,39-41], it has identified a biomarker for prostate cancer that it is currently progressing through clinical validation trials and have highlighted a number of other potential biomarkers, for diseases such as cancers and Alzheimer's disease, that may have failed to translate in previous studies due to alternate analytical approaches. While focus in proteomics has centred around MS, the potential to

refine and optimise DIGE approaches for biomarker identification and assay of proteomic signatures offers the required precision to bring proteomic and PTM biomarkers to the clinic for precision medicine.

Precision medicine has delivered effective therapeutics and much hope for some patients, yet the identification of actionable molecular targets to pinpoint future therapies is essential to this field. Analysing modified proteins as signatures of disease offers a range of largely unexplored potential biomarkers and is beginning to show promise in this field. It is well documented that glycans on the cell surface are fundamentally altered in tumour cells and that these changes happen early in the development of cancers, making them powerful early diagnostic markers. The specific changes in tumour cell glycosylation patterns determine the immune-inhibitory properties of the tumour, often allowing tumour cells to evade immune detection and prevent the arousal of an anti-tumour response. Strategies that prevent the interaction of tumour-associated glycans with inhibitory immune receptors could, therefore, serve as novel immune checkpoint inhibition therapies[36]. Recent research has shown that modification of tumour cell glycosylation patterns, through metabolic mimetics or glycosylation enzymes conjugated to tumour-targeting enzymes, reduced tumour growth and activated T-cell mediated anti-tumour responses[43,44]. The importance of considering patient PTM profiles was further highlighted in the trials of the vaccine Biomira (Theratope) which targeted anti-tumour associated glycan for the treatment of metastatic breast cancer[22,23]. During Phase II trials, a significant increase in patient survival was observed[23]. However, Phase III trials failed to reproduce these findings, probably owing to heterogeneity in the expression of the tumour PTM profile, which was not evaluated prior to patient selection[26,36].

Delivering biomarkers for accurate disease diagnosis and treatment is essential to precision medicine. While omics-based approaches have produced many effective biomarkers, particularly within the field of genomics, the proteomics field is not currently as advanced in translating findings to the clinic. A large part of this may be due to the requirement for refinement of proteomic profiling methods to identify and quantify clinical biomarkers and the vast complexity of the proteome in comparison to more static biological library of the genome. Understanding and being able to accurately analyse PTMs is an important challenge within this area that once we begin to meet, via improved MS protocols or alternative techniques such as DIGE, could begin to deliver actionable clinical biomarkers for improved precision medicine across the spectrum of disease. DDW

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