

Review

Biomarkers in rheumatology, now and in the future

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Abstract

This review examines the biomarker development process by using rheumatic disorders as the disease model for discussion. We evaluate the current role of biomarkers in the practice of rheumatology and discuss their likely role in the future. We define the essential components of the biomarker development pipeline and discuss the issue of fitness for purpose, i.e. what the biomarker(s) might offer in a clinical setting. As a component of this review we also highlight several emerging technologies that are beginning to provide practical solutions to support biomarker validation. In the process, we highlight some scenarios where additional biomarkers would add considerable value to clinical practice, and we review appropriate methods for each. We also emphasize some important but infrequently discussed considerations, including the need for protein variant verification. Ultimately, the adroit application of the methods of proteomics will transform the practice rheumatology and allow personalized clinical practice to become a reality.

Key words: arthritis, biomarker validation, proteomics, verification, validation, targeted analysis, protein microarray, mass spectrometry.

Introduction

Biological markers are widely used in medicine and can provide an objective measure of normal and pathogenic processes or pharmacological responses to a therapeutic intervention. By the term biological markers [or biomarkers, see supplementary data for glossary (including proteomic terms) available at *Rheumatology* Online] we mean an objective molecular indicator or surrogate of pathological processes that possess diagnostic, prognostic or predictive utility. These are distinct from clinical markers that rely on physical variables or symptoms such as joint count, pain assessment or radiological findings.

Biomarker examples include cardiovascular risk assessment through cholesterol checks; pituitary and target gland hormone determinations to assess endocrine function and dysfunction; haemoglobin A1c (HbA1c) evaluations to monitor blood glucose levels in diabetic patients; liver function tests in liver disease; and prostate-specific antigen determinations to assess prostate cancer risk. Not surprisingly, there is considerable interest in developing additional clinical biomarkers in medicine; however, the path from their discovery to routine adoption is commonly painstakingly complex and slow.

There are many obstacles that impede the biomarker development process. Although rarely discussed, the reality is that there are an abundance of candidate biomarkers available, but very few have undergone extensive validation.

In this review, we consider how biomarkers are used in the practice of rheumatology, how they might be used in the future, and we illustrate and discuss the significance of major barriers to their widespread clinical adoption. These challenges to new marker development and commercialization include factors such as study design, pre-analytical variables, data interpretation, bioinformatics, validation, ethics and commerce. While our focus is on protein biomarkers and proteomic methods in rheumatology, the principles we discuss are generally applicable to other analytes and areas.

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TABLE 1 Biomarkers routinely used in the diagnosis and treatment of arthritides

Marker	Molecular class	Example application
Creatinine	Metabolite	Creatinine is used together with liver enzyme determinations (AST and ATL) as an index of drug toxicity and kidney function [1].
CRP	Protein	A plasma protein routinely measured as a non-specific index of acute inflammation. CRP levels are typically integrated into clinical response scores, such as DAS-28, which help to titre drug dosage [2].
ANA	Protein autoantibody	Used along with other tests, ANA helps with the diagnosis of arthritides. ANA titres and nuclear stain patterns can vary between patients depending on the condition (~95% of SLE) [3]. Specific subsets of ANAs can be used to distinguish the type of autoimmune disease, e.g. SS. ANA positivity increases the risk of eye disease in JIA patients.
RF	Protein autoantibody	RF antibodies target the Fc region of IgG and are detected in 60–80% of RA patients, though are present in other inflammatory and CTDs [4]. The presence of RF early in the course of RA is associated with more active disease [5].
Anti-CCP antibodies	Protein autoantibody	Anti-CCP antibody positivity predicts the development of RA and may occur long before the onset of symptoms [6]. Anti-CCP is associated with severe erosive disease and can predict disease progression in RA patients [7].
Anti-dsDNA antibodies	Protein autoantibody	Anti-dsDNA antibodies are highly specific for the diagnosis of SLE with a specificity of 95%, but with a low sensitivity of <60% [8].
<i>HLA-DR4</i> or <i>HLA-DRB-1</i>	Nucleic acid gene	HLA genes have been found to be associated with RA. This association is particularly strong for <i>HLA-DRB-1</i> alleles that share a similar amino acid sequence known as the shared epitope [9]. The presence of these alleles both increases the risk of RA and associate with more severe disease [10].

Biomarkers currently used in the practice of rheumatology

The biomarkers currently used in the practice of rheumatology can be subdivided into three distinct molecular classes: nucleic acid, proteins and metabolites. Some of these tests and their applications are included in Table 1.

This is not an exhaustive list, and while an invaluable armamentarium, it has limited clinical utility and performance. With the exception of anti-CCP autoantibody and S100 proteins, the list has remained essentially static for the past 5–10 years. Additional potential biomarkers have been reported, but few, if any, have been adopted in routine practice. There is, however, no dispute that better tools are urgently required for both objective diagnosis and optimal management of RA.

The future: additional markers and rheumatology practice

Several areas of medicine lack objective, quantitative measures of a disease and its response to therapy, and the inflammatory arthritides are no exception. Below, we define three distinct stages in the clinical progression to chronic inflammatory disease and outline where and how biomarkers could aid in managing the RA patient.

- (i) The pre-symptomatic stage: here asymptomatic individuals, especially those with a strong family

history of RA, could be screened prior to disease onset. If the biomarker is specific and sensitive enough, such patients can be identified and monitored for the first signs of disease. Many patients with RA are not diagnosed for many months, indeed years, from the onset of symptoms due to the lack of gross clinical or laboratory findings. If we had a robust screening tool that identified these patients, much of the damage that occurs very early in RA could be avoided. Biochemical markers could remove uncertainty in detecting the disease in its early stages and would allow early and appropriate intervention. Such early detection could help clinicians minimize or even halt disease progression, reduce morbidity and mortality, and markedly lower the costs of health-care delivery in rheumatology.

- (ii) The early clinical stage: in the early stages of rheumatic diseases patients may be symptomatic but there are insufficient clinical or laboratory findings to confirm the diagnosis. A few biomarkers exist, which are helpful in predicting outcome at a population level, such as RF [11] and, more recently, anti-CCP antibodies for the diagnosis of RA (Table 1) [6]. IgM RF is useful in the diagnosis of RA; however, the sensitivity and specificity are relatively low. At a population level its presence does identify a group of patients whose disease will run a more aggressive course. However, ~15% of patients who are RF negative also have aggressive disease.

Anti-CCP antibodies are at least as sensitive as IgM RF in identifying patients with early RA. They are, however, more specific, and thus useful in patients with early, clinically undifferentiated arthritis [12]. Anti-CCP antibodies can identify patients likely to develop destructive erosive disease in the future. They are therefore very useful to the clinician, enabling them to instigate a more aggressive treatment plan early in the course of the disease [13]. However, the relatively low sensitivity and specificity of their assay offers relatively low diagnostic sensitivity, and this means that a significant minority of patients with aggressive disease cannot be identified [14, 15]. In recent years, a serological point-of-care test evaluated for the early detection of RA has been developed. This assay combines the detection of RF and anti-mutated citrullinated vimentin (MCV) for diagnosis of RA and shows a sensitivity of 72% and specificity of 99.7% [16]. While the sensitivity remains too low, it is a substantial improvement on RF status and is one of the first composite biomarkers to be used in RA. While not yet in widespread use, such approaches are undoubtedly the way forward. While RF and anti-CCP antibodies are useful in adult arthritis, they are of less use in a other inflammatory arthritides of adulthood, such as PsA and AS, or for the vast majority of children with JIA [17, 18]. ANAs are a family of antibodies directed against moieties of the cell nucleus. They are present in ~5% of the healthy population; however, they can be detected in up to 95% of patients with certain autoimmune rheumatic diseases such as SLE. However, their specificity is low, being found in a variety of diseases, including chronic liver and lung disease [19, 20]. Nonetheless, the determination of ANA status is useful to the clinician in directing them towards the possible diagnosis of an autoimmune disease. But ANA status is of little use in isolation. In contrast, antibodies against dsDNA are of enormous use to clinicians, as they are virtually diagnostic of SLE [21]. Unfortunately, their sensitivity is very low (30–60%). Thus, in their absence the clinician is required to use a collection of clinical and laboratory findings to confirm the diagnosis using internationally agreed criteria such as those developed by the ACR. ANAs are also of some use in children with JIA. Those who are positive are at greater risk of developing uveitis. However, the test is neither sensitive nor specific enough to be used as a definitive screening tool, and thus regular clinical examination is still required [22]. HLA-B27, a Class I surface antigen in the MHC, presents antigenic peptides to T cells. HLA-B27 is strongly associated with ankylosing spondylitis (AS) [23–25] and other associated inflammatory diseases such as PsA and arthritis observed in IBD. Some 95% of patients with AS express this antigen. Thus, clinicians diagnose this condition in the absence of HLA-B27 with considerable caution. Some 7%

- of healthy Caucasians express it, and thus AS is not diagnosed without radiological evidence of sacroiliitis.
- (iii) The disease management stage: early commencement of effective therapy is essential if joint damage and other complications are to be avoided. Historically, monitoring response to treatment is a composite of clinical findings and laboratory markers such as ESR, CRP and composite clinical and laboratory tools such as the DAS-28 [26], which have been shown to be useful in helping clinicians decide when to change patients treatment regimen and monitor response to that treatment. However, disease response can take many months, indeed years. Thus by the time the patient's disease is deemed unresponsive, substantial joint damage can have occurred. The identification of biomarkers that would predict disease response would have an enormous impact on outcome. Treatment may also be discontinued due to poor tolerability. Identifying such patients (personalized medicine) in advance would improve patient care and reduce stress. Biologic drugs as third-line therapy, such as anti-TNF, have revolutionized the treatment of rheumatic diseases, and systematic reviews have confirmed their efficacy and relative safety [27]. However, these drugs are extremely expensive. Months of treatment can be required before the clinician knows whether they are effective. This is both costly and inefficient. The identification of biomarkers that would predict the response of individual patients to these expensive agents would help patients, clinicians and funding agencies alike. Finally, there are concerns associated with the use of biological therapies, i.e. the risk of life threatening infections and more worryingly the long-term risk of malignancy [28]. The ability to identify such patients in advance would protect them from such serious adverse reactions.

Why target protein biomarkers?

Although genomic and transcriptomic methods are powerful, they do not predict downstream events. Specifically, they cannot predict what protein forms will be expressed in a particular tissue or biofluid, nor can they reliably estimate the levels of expressed protein [29]. Just as importantly, protein modifications including a plethora of post-translational changes are not evident upstream. As discussed later, proteins may require cleavage of a specific sequence to become biologically active, additional sidegroups may be added at specific amino acids, including phosphorylation, to propagate signal transduction or glycosylation to transport the molecule to a specific site [30]. It is the gene products themselves, viz. proteins that contribute directly to physiological or pathological change, and as such, they provide the best clues to function in health and dysfunction in disease. The analysis of hundreds (if not thousands) of proteins

by proteomics is therefore potentially the most illuminating of all multiplexed omics strategies.

Proteomics in practice, however, is an imperfect discipline, and although its methods are rapidly evolving, it is important to recognize that past and present approaches have significant limitations. Notably, the currently available discovery tools offer inadequate precision, sensitivity, specificity and throughput. An example of a low-throughput proteomic platform is classic liquid chromatography separation of a single complex biological sample, coupled with electrospray ionization generation of mass spectra. These limitations constrain study design (e.g. small sample numbers) and outcome (e.g. the errors on quantitative measures). Regardless of these shortcomings, proteomic methods have been widely adopted and have generated many potential candidate markers.

The proteomic biomarker development pipeline

There is no dispute that new biomarkers would advance the diagnosis and management of rheumatic diseases. The ongoing challenge, however, is not only how to discover candidate markers, but also how to validate them, i.e. define their performance characteristics when adopted in a routine clinical setting.

A major impetus for increased interest in biomarkers has been the introduction of the omics technologies. In a single study, these allow interrogation of hundreds (or thousands) of independent variables, such as genes, mRNA, metabolites or proteins, and given the volume of information generated from such studies, many have anticipated candidate biomarkers would flow quickly from each new investigation. The reality, however, is otherwise. Comparing the levels of hundreds (or thousands) of data points in several distinct groups, especially when the sample numbers are small, gives rise to many apparent differences, only some of which are related to biology. Chance alone gives rise to many apparent distinguishing features—the challenge is identifying the biologically relevant differences and ignoring the others.

For example, consider a proteomics experiment in which 200 proteins are measured simultaneously in control and test samples. At $P < 0.05$, 10 proteins will appear to be different by chance alone and unrelated to the treatment or condition. This consideration is not presented to undermine the value or utility of omics research, but rather to underscore the importance of applying appropriate statistical analysis to such large P and small n datasets and verifying any observed differences in follow-up studies. In more conventional scientific studies, it is typical to examine a single dependent variable, run replicates and to use standard statistical approaches to analyse the outcomes.

Contrary to what was hoped, the omics methods do not provide a fast track to biomarkers or shortcut the scientific process. They do, however, allow an investigator to operate independently of existing knowledge and to be less

dependent on insight, instinct or experience. A single omics study can provide data from which dozens of testable hypotheses can be formulated or, put another way, it can identify dozens of biomarker candidates (Fig. 1). Accordingly, the validation of each candidate biomarker is analogous to hypothesis testing where the investigator sets out to falsify (or disprove) the claim that candidate x is a valid biomarker in defined clinical scenario y .

Biostatistical considerations

Because proteomic studies of clinical samples generate large multivariate data sets, robust bioinformatic methods are required to find reproducible differences that correlate with a clinical outcome, e.g. response to biologic therapy, and are independent of the influence of experimental bias, over-fitting and statistical chance.

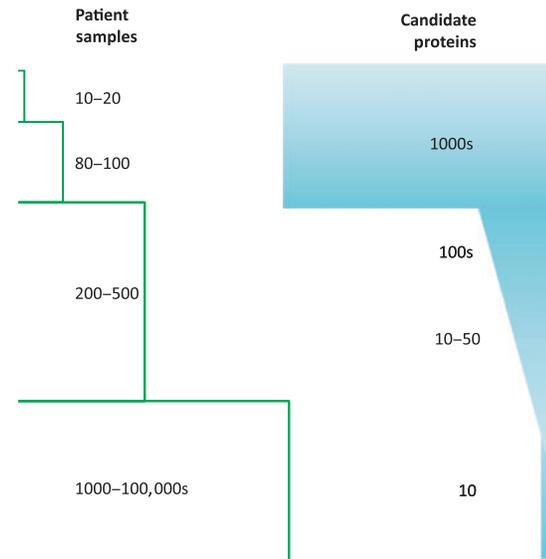
Bias, or any discrimination occurring due to a non-biological signal, can potentially confound discovery. For example, spurious results may arise because of differences in how patient samples are collected, e.g. type of blood collection tube, time taken to freeze sample or the order in which the samples are analysed. Over-fitting occurs as an artefact of regression analysis when many variables are fitted to a limited set of outcomes. The discriminating pattern or signature then becomes an artefact of the patient cohorts. To resolve issues of bias, statistical analyses must consider the biology of the system being analysed and take into account the assumptions and limitations of the methods [31].

False biomarker leads are common and therefore rigorous validation is essential. The false discovery rate can also be calculated to grasp the level of false-positive biomarker leads [32–34]. Additionally proteins displaying the most dramatic changes may appear to be useful biomarkers; however, it is important to rationalize their specificity to the pathology of interest. For example, acute-phase proteins are frequently identified across a wide range of chronic disorders, including arthritis and cancer, but clearly they are not specific to any one disease [35].

Given the issues noted above, it is advisable to validate on independent sample sets, incorporating analysis by orthogonal methods [36]. The statistical significance of a selected proteome classifier gives an incomplete estimate of its predictive ability and potential clinical utility. The number of true and false positives or negatives should be presented, allowing an estimate of sensitivity and specificity. This reveals clinically relevant information on how the classifier performs in each outcome category. Depending on the clinical question there may be multiple outcome measures that are not amenable to a simple binary classification where one can define false positives and negatives. Lists of statistical tools and recommendations for their application have been reported [36–38]. Statistical evidence of prevalence and analytical limits of detection of a specific group of isoforms should then direct the study towards validation of candidate biomarkers in a much larger group of multi-centre patient populations.

Fig. 1 Biomarker pipeline. Table describes the aim, the likely analytical platform and associated characteristics of each phase in an ideal biomarker discovery pipeline through verification to validation and final pre-market approval. The schema represents the increase in patient samples and decrease in candidate protein numbers as a biomarker study moves from discovery (two-step) through to the validation phase. 2DE: two-dimensional gel electrophoresis; DIGE: difference in-gel electrophoresis; LC-MS: liquid chromatography associated with mass spectrometry; IVDMA: *in vitro* diagnostic multivariate index assay.

Phase	Aim	Platform	Accuracy/precision	Sample turnover
Candidate discovery	Relative, unsupervised	2DE, DIGE, LC-MS	Low	Low
Specificity verification	Qualitative, PTM isoform targeted	MS/MS	High	Low
Sensitivity qualification	Quantitative, prevalence, classifier Test	ELISA, MRM	High	Medium
Clinical validation	Classifier validated, standardized, FDA approved IVDMA	MRM, high content array	High	High



Protein isoform considerations

Protein modifications are common but are frequently overlooked, especially during the assignment of protein identity in the discovery phase. Among the most significant modifications are covalent alternations to amino acids (e.g. phosphorylation, nitration or redox changes) and covalent addition of large groups (e.g. glycosylation). These modifications can have dramatic effects on protein function and may play a significant role in a range of arthritides and autoimmune disorders. Accordingly, changing levels of a modified protein may represent a better biomarker than changes in the protein's expression itself. For example, alterations in the levels of naturally occurring glycosylation motifs can serve as a marker of inflammation, lymphocyte tolerance and senescence in arthritis [39], viz. increased branching of sugar moieties on α -1 acid glycoprotein can act as biomarkers of inflammation, whereas decreased branching of T-cell receptor affects the development of Th1/Th2 cells, increasing susceptibility to autoimmunity [40, 41].

Recent evidence suggests that oxidative modifications to the proteins S100A8 and S100A9 shifts function from macrophage and neutrophil activation in inflammatory arthritis towards a protective role [42]. In this case, the modification appears to serve as a regulatory switch. Before a disease becomes apparent clinically, it is likely that disease-specific biochemical changes occur that underlie or reflect the pathophysiology. Alterations in protein isoforms may comprise part of the milieu of such biochemical changes and thereby serve as biomarkers.

For example, a recent study of diabetes patients revealed that, within a cohort of 96 individuals, an average of three variants of each protein were observed; a further eight variants were observed across 1000 individuals [43]. This highlights the importance of accounting for protein micro-heterogeneity across patient populations and the correlation of prevalence with specific disease outcome sub-groups (Fig. 2).

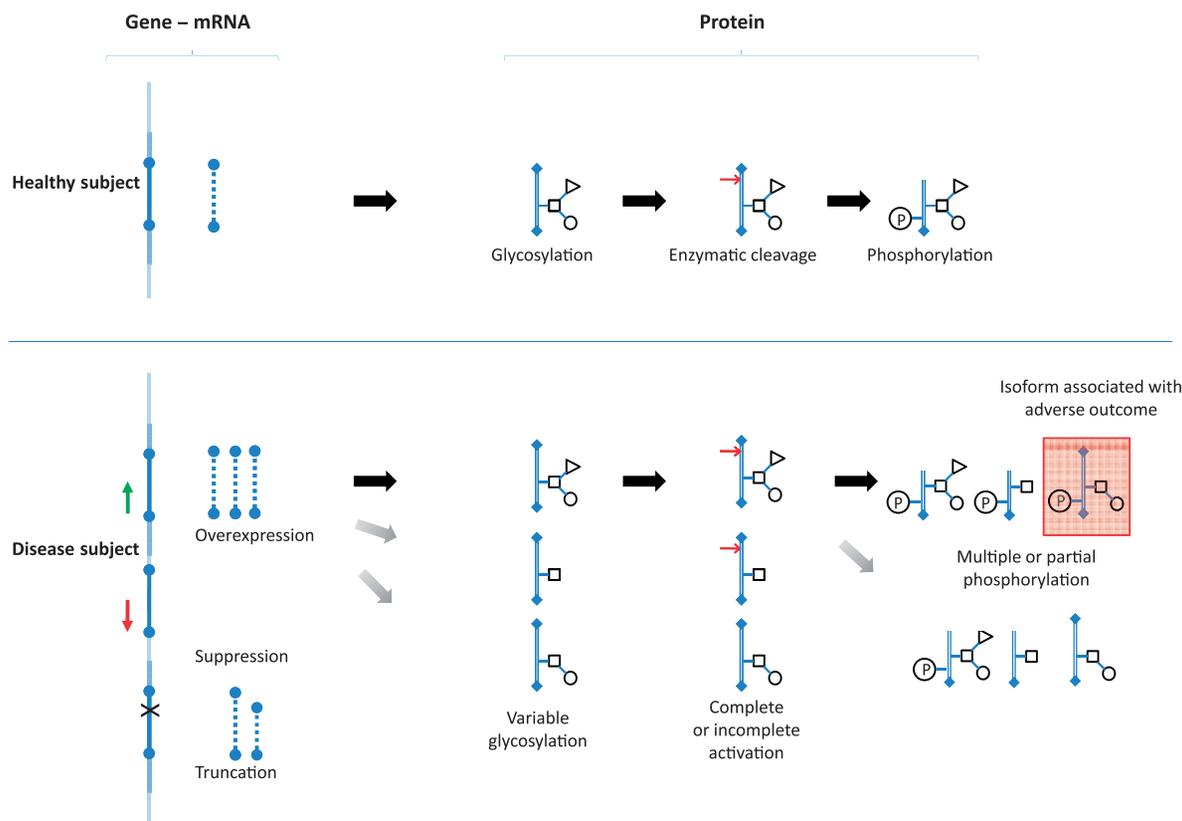
Emerging tools for targeted biomarker validation

The biggest challenge in proteomics remains independent validation of changes discovered in observational investigations. Traditionally, validation has been undertaken by antibody-based approaches, including western blotting, ELISA and immunohistochemistry. However, despite major efforts to generate proteome-scale panels of suitable antibodies [most notably the impressive Human Protein Atlas initiative (<http://www.proteinatlas.org/index.php>)], this remains a slow process. It requires antibody generation and characterization to establish specificity and utility in different assay formats.

Multiple reaction monitoring

Antibody-independent strategies are also highly desirable. In recent years, significant advances have been made in the measurement of protein expression using multiple reaction monitoring (MRM) on triple quadrupole (QQQ) mass spectrometers [44, 45]. MRM is an approach for protein

Fig. 2 Protein isoform verification. A depiction of the possible qualitative and quantitative changes in protein isoforms encountered in a disease state. The illustration of an isoform of the given protein associated with a specific adverse outcome demonstrates that it can only be detected by high-resolution proteomic strategies that account for variance in post-translational modification. Conventional genomic and antibody-based methods will only pick up on a change in expression of recognized transcripts or epitopes, giving a high likelihood of missing the significance of the isoform prevalent in a particular disease outcome.

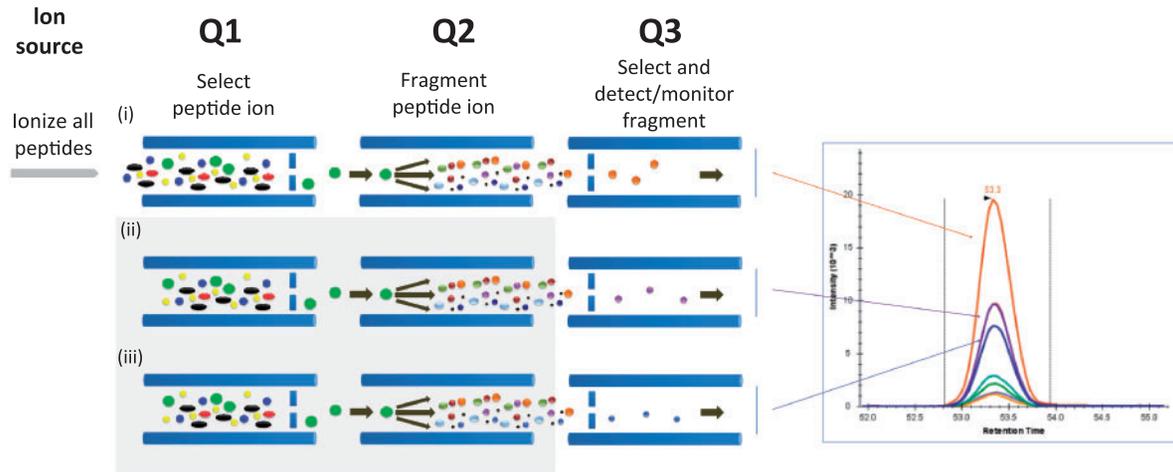


quantification at the peptide level that is growing rapidly in popularity, as it can reliably quantify peptides in complex mixtures in a multiplexed fashion. In an MRM measurement, a protein is measured by identifying one or more peptide ions generated by trypsin digestion of the protein that are of unique mass, i.e. could only have originated from the protein of interest—these are termed proteotypic peptides. Proteotypic peptides (and hence their protein of origin) can be measured in a sample that has been treated with trypsin in a targeted manner. Thus, the proteotypic peptides are individually pre-selected in the first quadrupole (Q1) of a QQQ instrument so that only the peptide ion of interest is transmitted to the second quadrupole (Q2), where it is induced to fragment [46]. Then in the third quadrupole (Q3), some of the resultant peptide fragments are selected for transmission to the detector (Fig. 3). MRM supports the simultaneous measurement of multiple proteotypic peptides, and synthetic mass variants spiked into samples in known amounts can transform the analysis from a qualitative to a quantitative assay. Hence, the strategy enables the absolute quantification of multiple proteins [47, 48]. When MRM is combined

with immunoaffinity purification and internal peptide standards, for example in stable isotope standards and capture by anti-peptide antibodies (SISCAPA) assays, detection is in the sub-femtomolar range. The combination of MRM, chemistry and software to aid with the selection of suitable proteotypic peptides, has provided the opportunity to rapidly develop quantitative multiplexed assays of protein expression and post-translational modification that are both highly specific and sensitive [45].

In a relatively early demonstration of peptide MRM, one of us (S.R.P.) developed MRMs to simultaneously quantify the expression of 16 cytochrome P450 enzymes—proteins important in determining susceptibility to adverse drug reactions [49]. Of relevance to rheumatology, previously a method was described for the MRM assay of CRP as a means of differentiating erosive from non-erosive RA patients [50]. The same research team subsequently applied the same MRM technique to measure elevated levels in SF of six additional members of the S100 calcium-binding proteins associated with an erosive subtype of RA [51].

Fig. 3 MRM mass spectrometry. In protein MRM, one or more peptides of unique and known mass (proteotypic peptides) are pre-selected in the first quadrupole (Q1), induced to fragment in Q2 by collisional excitation with a neutral gas in a pressurized cell and some of the resulting product ions (fragments) are selected for transition to the detector in the third quadrupole (Q3). Fragments and their resultant spectra are illustrated for three different user-selected fragmentation reactions (i–iii). In an MRM assay, a predefined precursor ion and one of its fragments are selected by the two mass filters of a QQQ instrument and monitored over time for precise quantification. A series of transitions (precursor/fragment ion pairs) in combination with the retention time of the targeted peptide can constitute a definitive assay [43].



Nucleic acid programmable protein arrays

The production of antibodies against self-antigens (autoantibodies) is a characteristic feature of many autoimmune diseases. At a clinical level, tests for specific autoantibodies, such as ANA positivity, are routinely employed to aid the diagnosis and track the progress of these diseases. Traditionally, autoantibodies have been identified with a one-antigen-at-a-time, hypothesis-driven approach using methods such as IF and ELISA.

Microarrays provide a particularly effective platform for the systematic study of thousands of proteins in parallel because they are sensitive and require low sample volumes [52, 53]. Protein microarrays involve the display of thousands of different proteins with high spatial density on a microscopic surface. Protein microarrays have been applied to autoimmune biomarker studies focused on pre-symptomatic screening and diagnosis, clinical outcome prognosis and therapeutic response prediction [54–63]. In a recent study, a strong correlation was observed between 768 autoantibodies in paired plasma and SF samples from patients with juvenile arthritis (Fig. 4).

Nucleic acid programmable protein array (NAPPA) is an innovative method to produce protein microarrays, where cDNAs encoding proteins of interest are spotted onto activated surfaces and proteins are produced *in situ* using mammalian *in vitro* expression systems [64, 65]. The freshly made protein is captured by co-spotted antibodies specific for a tag encoded at the end of the amino acid sequence. This approach circumvents the labour and cost considerations associated with conventional spotting of labile recombinant proteins into arrays.

NAPPA technology recently revealed that AS patients' autoantibody responses were targeted towards connective, skeletal and muscular tissue, unlike those of RA patients [66].

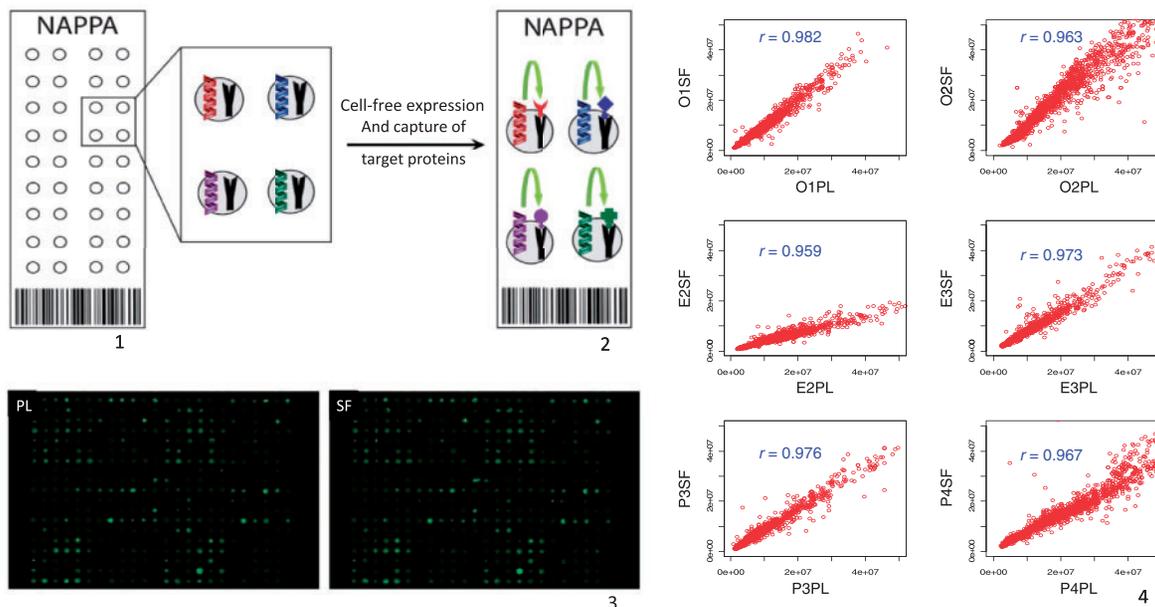
Proteomic profiling methods

Intact protein profiling across clinical cohorts gives a glimpse into the degree of variation evident in a single gene product [67]. The same approach may be useful in the study of arthritis. Mass spectrometry-based techniques can potentially distinguish these physical and structural variations and allow the relative abundance of one isoform to be determined [68]. In contrast, these variants would be overlooked by conventional ELISA methods (Fig. 2). A brief description and recent application of such techniques follows.

Matrix-assisted laser desorption ionization/surface-enhanced laser desorption ionization profiling (Immuno-MALDI)

The matrix-assisted laser desorption ionization (MALDI) mode of mass spectrometry allows the soft ionization of complete proteins that are liable to fragment under conventional ionization methods. The type of a mass spectrometer most widely used with MALDI is the time-of-flight (TOF), mainly due to its large mass range. Purifying a protein from a clinical sample by immunoprecipitation can greatly reduce the complexity of the proteome being analysed. In one approach, purified polyclonal antibodies that capture the target protein isoforms can be immobilized onto sepharose beads packed within a pipette tip or fret [69]. Eluted proteins can then be spotted on a MALDI

Fig. 4 NAPPA. (1) NAPPA spotted with genes of interest; (2) all proteins are tagged at the c-terminus to ensure only full-length translated proteins can be captured *in situ* by co-spotted anti-tag antibodies. Proteins are expressed just in time for assay, which eliminates the concern of protein stability. (3) Paired images for NAPPA with randomly selected 768 genes probed with the plasma (PL) and the SF sample from the sample patient with juvenile arthritis. Antibodies in patient samples bind to their antigen targets on the array and are detected by Alexa 647-conjugated goat anti-human IgG. (4) Scatterplots of reactivity on NAPPA between 10 paired plasma and SF samples from juvenile arthritis patients. Median correlation is 0.966.



target plate and spectra obtained. For example, some recent MALDI profiling applications have demonstrated the ability to diagnose early RA and hypertension and distinguish active SLE [70–72]. Glycosylation heterogeneity of selected inflammation-associated molecules, such as serum amyloid and vitamin D binding protein, has been investigated in cancer and diabetic patients [73, 74].

As a modification of MALDI, surface-enhanced laser desorption ionization (SELDI) methods can be used to target lower molecular weight proteins (<20 kDa) to differentiate arthritides and therapeutic response [75, 76]. The technology is currently being developed to affinity capture the protein of interest directly to the mass spectrometry target plate [77].

Biomarker research and grant funding

Although proteomics has been full of promise, few validated biomarkers have made their way into the public domain and even fewer influence clinical practice. There is little doubt that validation is a serious bottleneck in the biomarker development process. While there is abundant discussion of approaches to discovery, the tools for validation and their applications have received little attention. It is very often difficult to receive funding from traditional grant programmes to validate markers: funding agencies balk at the prospect of funding a re-measurement of the same entity in larger independent cohorts. Additionally,

the continuum from discovery through to validation is tedious and extends well beyond the time-frame of a typical research grant. In fact, the time from initial discovery to routine use can take up to a decade [78, 79]. A recent example illustrates the 7-year journey from discovery to FDA approval for the multivariate diagnostic test produced by Vermillion as OVA1, used to screen ovarian cancer patients [80].

Similarly, when validation fails it is difficult for academic investigators to publish these negative results; when validation succeeds, the emphasis frequently shifts to commercialization rather than publication.

Conclusions

While there is widespread recognition of the value of biomarkers, scientific progress is slow. Over the years, biomarkers have sometimes been the centre of exuberant hype, prompting excessive or unreasonable expectations. In addition, the use of biomarkers as surrogate endpoints have led to some public failures when they were felt to be falsely reassuring or too alarming, creating general scepticism among some scientists.

Currently there are no FDA-approved proteomic tests for arthritis. Although there is little doubt that such tests could help the diagnosis and treatment of arthritis, it is a major clinical and financial challenge to develop, validate and market them. Robust validation data including

evidence of sensitivity, specificity and correlation to the existing limited set of clinical or laboratory criteria are necessary to support clinical utility. The measurement of specific proteins that flag a particular patient's status could add objectivity in circumstances where the clinician currently relies on clinical judgement alone. We have discussed three key areas for consideration, which if addressed after initial discovery work could provide solid evidence of their clinical utility and commercial viability: (i) limiting bias in study design, (ii) thorough protein isoform verification; and (iii) modes of orthogonal and targeted validation.

Rheumatology key messages

- Longitudinal cohorts followed from diagnosis are desirable for the development of prognostic/predictive markers.
- Standardized methods of sample processing should be adopted to avoid pre-analytical variation in biomarker levels.
- Post-translational modifications of biomarker candidates should be measured.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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