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# **Biomarkers of Inflammatory Arthritis and Proteomics**

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Additional information is available at the end of the chapter

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## **1. Introduction**

Autoimmune diseases are systemic and organ-specific inflammatory conditions involving a cell-mediated immune response against self tissues. Whilst it is known that they are characterized by autoantibodies in both the systemic fluid and tissues [1, 2], the detailed aetiology and pathogenesis of auto-immune diseases are still poorly understood [3, 4]. These types of diseases occur when self molecules, often unknown antigens (auto-antigens), are seen by the immune system as non-self and are thereby attacked immunologically by the production of autoantibodies against them. In this process, the immune system mistakenly reacts with the body's own cellular gears as if they were foreign antigens. The clinical presentations of autoimmune diseases vary with the disease course and delay in diagnosis as well as inappropriate therapy, increases tissue damage [3, 5].

Inflammatory arthritis (IA), an autoimmune disorder of the joint tissue, is characterised by influx of white blood cells in the joint fluid. The disease often progresses to articular destruction, joint ankylosis (stiffness of the joint) and functional disability [3]. IA is a chronic disease, persisting as it often does for a long time, and some forms of the condition are systemic affecting many tissues and organs other than the joint and skin. IA is a significant cause of disability in those over fifty-five years of age, and is among the leading conditions restricting an individual's capacity to work. The healthcare, socio- and pharmaco-economic challenges of IA are significant. The most important issues for healthcare include: i) recognition and establishing an early diagnosis for disease; ii) identification of patients who are likely to develop a worse prognosis; iii) predicting and selecting therapies to which patients will respond; and, iv) understanding the balance between limited healthcare resources and the expensive disease modifying anti-rheumatic drugs (DMARDs) [3].

There has been much excitement about the potential of the omics technologies to deliver novel biological markers (biomarkers) of sufficient discriminatory power that they could herald an era of personalised medicine [6]. Personalized medicine being a medical approach that customizes healthcare; tailoring decision to individual patients based on genetic, proteomic or other information. Accurate prediction is essential for personalized medicine to ensure that therapy is given to those individuals who are likely to develop worse prognosis [7]. More specifically, the application of proteomics (the study of proteins), has been suggested to hold special promise for the discovery of clinically useful biomarkers [6, 8]. Biomarkers are characteristics that can be measured or evaluated to indicate a normal biologic process, a patho-physiologic condition, or a pharmacologic response to therapy. Biomarkers are also defined as measurable variables of how a patient feels or functions. [9, 10]. Proteomics is extremely powerful for both biomarker discovery and for the investigation of biochemical processes involved in diseases. At its most straightforward, proteomics involves the comprehensive determination of protein expression levels and hence enables pathway determination of cellular processes [11]. Several proteomic approaches have been applied to the investigation of autoimmune disorders including (i) autoantigen and biomarker discovery by 2-dimensional gel electrophoresis (2-DE) based separation of proteins and subsequent protein identification by mass spectrometry; (ii) protein microarrays for the characterisation of antibody responses; (iii) reverse phase protein arrays to analyze protein phosphorylation (iv) antibody array technologies to profile cytokines and other biomolecules; and (v) flow cytometric analysis of phosphoproteins [2, 3].

Here we review the role of autoimmunity in IA with emphasis on disease aetiology, pathogenesis, existing biomarkers, assessment of disease activity, autoantibodies capable of predicting disease outcomes and latest therapies. We then outline the application of proteomics to the discovery of protein biomarkers in rheumatoid arthritis. The processes and challenges involved in validating potential biomarkers and developing them to laboratory tests of clinical utility are also summarised. Finally, we discuss some future directions in protein biomarker research in IA that may support personalised medicine for this autoimmune disease.

### **1.1. Inflammatory arthritis: An autoimmune disorder**

Inflammatory arthritis is an autoimmune disease; it has the characteristic hallmark of activated immune cells that target self tissues. This auto-immunity is always as a result of complex interaction between genetic and environmental factors [3]. There are several forms of IA including rheumatoid arthritis (RA), psoriatic arthritis (PsA), juvenile idiopathic arthritis (JIA), ankylosing spondylitis (SpA) as well as the inflammatory form of osteoarthritis (OA). Cells involved in autoimmune IA include macrophages, T cells, B cells, fibroblasts, chondrocytes, and dendritic cells. It is known that the expression of key cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6) drives the inflammatory and destructive processes. TNF- $\alpha$  is a pro-inflammatory cytokine that is associated with fever and some other symptoms such as pain, tenderness and swelling, in several inflammatory conditions and over recent years has been a major target of treatment for IA [12].

In this review, emphasis is placed on RA, the most common form of IA. With its course clinically unpredictable, RA is associated with synovial inflammation in which the synovium, a thin

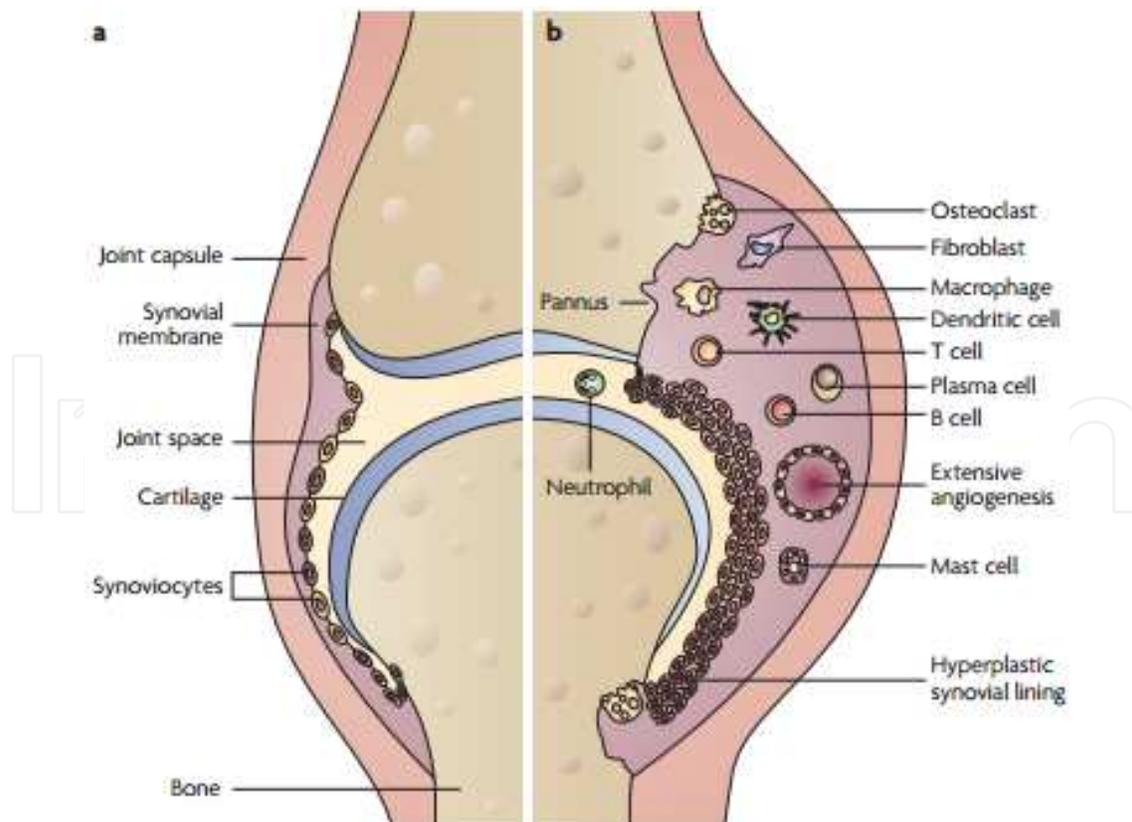
layer of tissue that lines the joint cavity, is the primary site of the cell-mediated inflammatory reaction [13]. RA has a very poor prognosis when compared to enthesal-based inflammatory conditions such as spondyloarthritis where the entheses - the site where tendons, joint capsules or ligaments insert into bone - are inflamed [14]. RA is often characterised by chronic inflammation of the joint that has been infiltrated by activated mononuclear cells. Inflammation is usually accompanied with swelling, pain and the destruction of articular cartilage, which ultimately lead to functional impairment of the affected joint [5, 15]. RA can hence be classified as an heterogeneous disease due to its different forms of clinical manifestations, serological abnormalities, functional impairment and joint damage [16, 17]. It has been reported that early and aggressive treatment of RA can prevent cartilage damage [3]. Before the introduction of 'biologic' therapies, the direct and indirect costs incurred for medical, social care and loss of employment experienced by RA patients and society at large were estimated to be \$98 million to \$122 million per million population in developed countries [18, 19].

## 1.2. The aetiology / pathogenesis of rheumatoid arthritis

The synovial membrane is the thin layer that lines the joint. It produces synovial fluid, which nourishes and lubricates the joint. Two cell types characterise the synovial membrane: macrophage-like and fibroblast-like synoviocytes. Although the pathogenesis and the aetiology of RA remain unclear, it is sometimes associated with genetic and environmental factors. Environmental factors associated with RA include cigarette smoking, alcohol, some reproductive factors in women, bacterial products, viral components and some other diverse environmental stimuli [12, 20]; the genetic factors are linked to the class II major histocompatibility complex (MHC) region on chromosome 6 and an association with the non-MHC gene (PTPN22-a protein tyrosine phosphatase that regulates T cell activation). Additionally, predisposing genes such as the HLA-DR4 allele are reported to be prevalent [18, 21]. Innate immunity is a primitive pattern-recognition system that leads to rapid inflammatory responses. In RA, innate immunity has been implicated through the engagement of Fc receptors by immune complexes and perhaps Toll-like receptors (TLRs) by bacterial products. Antigen-driven T cell and B cell responses may also participate as a result of either xenoantigen reactivity or, more likely, responses directed at numerous autoantigens. Evidence of autoimmunity, including high serum levels of autoantibodies such as rheumatoid factor (an antibody against the Fc region of other antibodies) and anti-citrullinated peptide antibodies, can be present for many years before the onset of clinical arthritis [20]. In some patients, worse prognosis of RA has been linked to the presence of rheumatoid factor and anti-CCP (antibody against citrullinated epitopes on post-translationally modified proteins). The proliferation of synoviocytes as RA progresses leads to the invasion of the hyperplastic synovial tissue and is responsible for the destruction of the underlying bone and the articular cartilage [21]. Cytokines and chemokines play an essential role in the angiogenesis and pathogenesis of RA [22, 23]. The expression of cytokines such as tumour necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-6, IL-15 and IL-17 during new blood vessel formation (angiogenesis) and inflammatory cell infiltration drives the inflammatory and destructive processes of this disease [21, 23]. The clinical presentation of RA differs from other forms of arthropathies with its characteristic symmetric polyarticular joint inflammation and destruction as well as its extra-articular manifestation (rheumatoid nodules/vasculitis) and the

presence of intracellular citrullinated proteins. The synovial membrane is affected in a number of ways which include architectural changes (neovascularisation, lymphocyte infiltration and thickening of the synovial lining layer) [23, 24]. Cardiovascular diseases, excess morbidity and mortality from myocardial infarction and allied disorders, high risk of lung diseases, coronary artery disease, lymphoma, infection as well as reduced life expectancy are associated with rheumatoid arthritis [16].

A model has suggested that in predisposed individuals, a stimulus or an infective agent binds to toll-like receptors on macrophages and peripheral dendritic cells; thereby triggering a rapid response from the innate immune system involving inflammatory mediators, cytokines, complements, neutrophils and natural killer cells. The migration of these cells to the joint leads to joint damage as a result of the actions of growth factors, proteases and activated osteoclasts. This damage is associated with the development of locally invasive pannus tissue [18]. The major joint destruction occurs at the pannus; this is the point at which the synovium meets the cartilage and the bone. The pannus is rich in macrophages and it is the major site at which irreversible tissue damage originates [25, 26]. The main cause of disability in RA is joint destruction that is characterised by progressive bone erosion [18]. As shown in Figure 1 below, infiltration of the joint by macrophages ultimately form the pannus that migrates into the bone leading to bone erosion. The inflamed joint compared to the normal joint is characterised by the influx of a number of inflammatory cells in the joint.



**Figure 1.** Normal healthy joint (a) and rheumatoid arthritic joint (b). [21]

In the healthy joint (a), a thin synovial membrane lines the non-weight-bearing aspects of the joint. While in the arthritic joint (b), the synovial membrane becomes hyperplastic and infiltrated by chronic inflammatory cells that develops into 'pannus', which migrates onto and into the articular cartilage and underlying bone causing bone erosion.

### 1.3. Current therapeutic targets and therapy in RA

Cytokine networks involving tumor necrosis factor, interleukin-6, and many other factors participate in disease perpetuation and can be targeted by therapeutic agents [20]. Disease-modifying antirheumatic drugs (DMARDs) have been used for decades to manage rheumatic diseases. Methotrexate is the most widely used disease DMARD. Other DMARDs include leflunomide, sulfasalazine, cyclosporine and hydroxy-chloroquine (20). However, in the past decade, biologic therapies such as fusion proteins and monoclonal antibodies have revolutionized the management of autoimmune IA. Biologics have provided more specific therapeutic interventions with less immunosuppression by targeting immune cells and key cytokines [21]. The biologics neutralize the actions of cytokines and proteins through human or chimeric monoclonal antibodies or by using a recombinant-antagonist form of the cytokine receptor [14, 27]. Example of such biologics is the TNF- $\alpha$  inhibitors. TNF- $\alpha$  being a key component in the cascade of cytokines induced in RA, is a target compound for treatment. To exert its effects, TNF- $\alpha$  bind to two receptors, the type 1 TNF receptor (p55) and the type 2 TNF receptor (p75), found on immune, inflammatory, and endothelial cells. TNF inhibitors were first licensed for clinical use in 1998; three have been approved for the treatment of RA. TNF inhibitors are introduced to patients with active disease who have not had a response to conventional DMARDs. Examples of the monoclonal antibodies include infliximab (Remicade), a chimeric human-murine IgG1 anti-TNF- $\alpha$  antibody administered intravenously. Infliximab is also cytotoxic for TNF-expressing cells; adalimumab (Humira) or certolizumab pegol (Cimzia). Humira is a recombinant humanized monoclonal anti-TNF- $\alpha$  antibody administered subcutaneously. There are also circulating receptor fusion proteins such as etanercept (Enbrel), a recombinant soluble p75 TNF- receptor fusion protein administered subcutaneously [18]. By inhibiting the action of TNF- $\alpha$ , the 'biologics' reduce the signs and symptoms of inflammation and stop the progression of joint damage. Therapeutic response in RA is assessed by clinical disease activity score 28 (DAS28) or ACR (American College of Rheumatology) criteria, structural (sharp or Larsen scores) and or functional evaluation standards (HAQ score). Treatment efficacy is usually estimated by comparing these assessments before and after treatment [27].

### 1.4. Disease activity and progression in RA

The terms 'activity' and 'severity' are usually used to characterize RA. Disease activity in this sense refers to the degree of overall inflammation measured by considering factors that include acute phase reactants, tender/swollen joints, pain, general impact, grip, strength and functional disability. Disease severity on the other hand, is more complex as it refers to the outcome or result of RA. Disease severity is measured by assessing radiographic abnormalities, indirect and direct costs, work disability, mortality and social

losses; generally elucidate the extent the disease has affected the patient and its effects. Severity explains the absolute social and physical damage resulting from RA as well as the rate at which the damage occurs [28, 29]. Informative characteristics for clinical outcomes can be broadly divided into prognostic or predictive biomarkers. Prognostic biomarkers show the expected clinical outcomes of patients such as progression or death. However, they do not inform the choice of therapy while predictive biomarkers would identify group of patients whose diseases are likely to be resistance or sensitive to therapy based on the biomarker status [30].

## 2. Assessment of disease activity and response criteria in RA

In practice today, the European League Against Rheumatism (EULAR) criteria are used to classify the disease activity in IA while the American College of Rheumatology (ACR) improvement criteria is the second method used for clinical evaluation of patients [31]. In RA, inflammatory activities cannot be measured using one single variable. The disease activity score (DAS) was developed to solve this problem [32]. DAS score provides important quantitative analysis in clinical research of RA and the score includes tender joint count, swollen joint count, C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR) level and the general assessment of the patient's health measured on a visual analog scale. At baseline and follow up, responses to treatment are assessed as low, moderate or high disease activity. DAS is best defined by statistical methods which include multiple regression analysis and discriminant analysis [31, 33]. A low disease activity is indicated by a DAS score  $<3.2$ , a moderate disease activity is indicated by DAS score  $3.2-5.1$  while a DAS score  $>5.1$  indicates a high disease activity [34]. These criteria are being used by the EULAR to define good responders, moderate responders and non responders to treatment as shown in Table 1 below. Advantages of the DAS include the following: its content is more informative than single variables, its values can be interpreted clinically and it has a continuous scale with a Gaussian (normal) distribution [32].

Disease Activity Level	Disease Activity Score (DAS 28) at Endpoint	DAS Improvement from Baseline		
		$>1.2$	$>0.6 \leq 1.2$	$\leq 0.6$
'Low'	$\leq 3.2$	Good response	Moderate response	No response
'Moderate'	$>3.2 \leq 5.1$	Moderate	Moderate	None
'High'	$>5.1$	Moderate	None	None

**Table 1.** The EULAR response criteria [32]

The ACR improvement criteria are used in clinical trials for the evaluation of RA patients. It is also referred to as the core data set. It involves swollen joint count, tender joint count, acute phase reactants i.e. ESR/CRP and the general health assessment questionnaire (HAQ score). The questionnaire assesses the pain, disability, and overall health of the patient. The ACR measures improvement in percentage of the changes in the criteria scales used. 20%, 50% and 70% improvement in any 4-6 of the scale used is an indication that the patient meets the criteria [34].

## **2.1. Clinical and biological markers used in practice for RA**

Clinically meaningful biomarkers may be based on proteins, genotypes, histology, metabolic patterns, or imaging techniques and they are ideal for early diagnosis, monitoring and prediction of therapeutic response. In a number of diseases and especially in RA, some patients do not respond to therapy at all and several others show diverse degrees of response. Therefore, biomarkers are urgently needed in the clinical setting (i) to select patients before treatment, (ii) to monitor the patients' response to the therapy as well as the disease activity, (iii) to classify patients into their different response categories (good responders, moderate responders and nonresponders) and (iv) to determine the OBD (optimal biological dose) for the drug [35-37].

Clinical markers are the physical symptoms or variables such as swollen joint count, tender joint count, pain assessment and radiological findings while biomarkers are molecular indicators of pathological processes as described earlier to assess diagnostic, prognostic and predictive features [38]. Biomarkers of RA can be broadly divided into two groups: Biomarkers of disease activity and biomarkers of joint damage.

Biomarkers of disease activity in RA are also referred to as 'momentary predictors' and include factors that change with time revealing the disease activity e.g. ESR and CRP [19]. Biomarkers of disease activity include cells, cytokines and acute phase reactants/proteins. Cells involved in RA synovitis exhibit increased number of macrophages in the synovium. B-cell depletion and the serum level of proinflammatory cytokines (IL-6 being the most abundant cytokine) can give the reflection of active disease. High Composition of synovial tumour necrosis factor (TNF) is also a biomarker of RA. Serum amyloid A, an acute phase protein, blood sedimentation rate as well as the presence of rheumatoid factor (RF) also reflects disease activity; as this can be used to calculate the disease activity score 28 (DAS28 i.e. 28 joint counts) [5, 17]. CRP, an acute phase protein has been successful to some extent to monitor early stage disease and progression after the commencement of therapy. CRP can be alternatively measured by calculating ESR [21]. However, some other factors have been found to influence the levels of both ESR and CRP rendering these as non-specific markers; these factors include anemia, aging and the presence of immunoglobulin such as rheumatoid factor [9]. Studies have shown CRP as a marker of different inflammatory disease activity [39]. As momentary predictors reveal outcomes, they require re-sampling to boost the accuracy [28].

Biomarkers of joint damage in RA are also referred to as 'cumulative predictors' and their outcomes are variable and worsen with time. These include radiographic erosion and func-

tional disability [28]. In RA, changes in bone and cartilage lead to joint damage. Spaces in the joint signify cartilage changes while erosion indicates bone destruction. Hence, either of these two changes can present a biomarker of RA joint destruction. Cartilage damage causes a change in the matrix composition of the cartilage thereby affecting the major proteins in cartilage: type 11 collagen (COL 2), aggrecan, and non-collagen and non-aggrecan proteins. The main structural protein in bone is the type 1 collagen and the non collagen protein in bone is the sialoprotein which is released during bone damage [17].

Some RA patients with active disease present with normal levels of some of these markers; this might be due in parts to genetics [40]. Due to lack of specificity, such markers might not be good for all RA patients; hence, a need for better biomarkers. The understanding derived from the disease biology has shifted the treatment strategy to targeted therapy. However, to develop, verify, validate and apply new and existing treatment successfully, there is a need to understand the difference and relationship between putative biomarker and treatment effect [30].

Notably, to date, there is no effective approach available to clinicians to predict which patient will respond to which therapy. For example, predicting which patients are likely to respond to TNF- $\alpha$  inhibitors would have significant value for the selection of patients whose condition warrants this high cost and 'high risk' treatment [41]. Therefore, there is an urgent need for new and better biomarkers in IA to improve diagnosis, support emerging targeted therapies, monitor drug activity and evaluate therapeutic response [1, 3].

In RA, any indicator of inflammation could be a biomarker [5]. These may include genes or products of gene expression, a cytokine, autoantibody, some acute phase proteins, tissue degradation product or tissue abnormality observed immunohistochemically in synovial biopsy. Sources of these biomarkers may include the serum, synovial fluid, urine, cells (lymphocytes such as the peripheral blood mononuclear cells (PBMC)) and tissues taken from the inflamed synovium [17]. RA biomarkers effectively used in practice are acute phase reactants (CRP and ESR) as well as autoantibodies Such as rheumatoid factor (RF), anti citrullinated protein antibodies (ACPAs) and anti-nuclear antibodies (ANA). These autoantibodies are capable of predicting disease outcomes and are used as laboratory markers to classify and diagnose RA. Autoantibodies are dependent on the organ or tissue affected as well as the severity of the disease. [42-44]. Highlighted below are the markers used in clinical practice for the diagnosis and prognosis of RA:

- Rheumatoid factor (RF): These are antibodies against the Fc region of other antibodies [21]. This IgM isotype of the serologic indicator of RA is useful in determining the auto immune status of the disease but it is not very sensitive to the changes in the disease activity level or specific as it could be present in patients with chronic infections, other immune diseases or in the elderly. The main use of which is a prognostic marker [45, 46]. RF predicts disease severity but it is not a good marker for early diagnosis. It is not very sensitive and does not vary stoichiometrically with treatment [29].
- C-reactive protein (CRP): RA increases the level of an acute phase reactant, CRP. This is a typical marker of inflammation and it has an association with cardiovascular risk

[16]. CRP is a plasma protein used for drug dosage titration as well as clinical response assessment [47]. Although CRP is a widely used biomarker of RA, it lacks disease selectivity [48]. CRP correlates with disease activity but does not predict the severity of the disease [29].

- Erythrocyte sedimentation rate (ESR): This contributes highly to disease activity but it is not a sensitive parameter and it can be easily affected by external factors such as age, fibrinogen level, gender, hypergamma-globulinemia, anaemia and RF [48]. Like CRP, it does not predict the subsequent severity of the disease [29].
- Anti-citrullinated protein antibody (tested as anti-cyclic citrullinated peptide (Anti-CCP) antibody): Recently, anti-CCP antibody has been effectively used for the diagnosis of RA [43, 47]. ACPAs are circulating autoantibodies against citrullinated epitopes on post translationally modified proteins. Citrulline is a non standard amino acid that originates from the enzymatic modification of deiminated arginine residue [46]. The citrullination/modification of arginine by deimination occurs physiologically during inflammation, apoptosis or keratinization. These proteins can be found either in the sera or in the synovium of RA patients. The presence of citrullinated proteins are associated with a worse prognosis of RA [21]. ACPAs are markers of RA with a specificity of 95-98% and a sensitivity of 70-80% [49]. As a result of their excellent diagnostic value, they are a better alternative to RF and they are widely used for the diagnosis of RA [45, 46]. The presence of this autoantibody in the serum precedes the onset of the disease and also linked to the pathogenesis of the disease. The isotypes of this antibody includes IgA, IgG1, IgG2, IgG3, IgG4 and IgM [45, 49]. Anti-keratin antibodies (AKA) and anti-perinuclear factor (APF) are examples of members of ACPAs. An example of anticitrullinated antibodies that has been detected in the sera of RA patients is the antibody against citrullinated vimentin. Vimentin, a protein that plays vital biologic role in contraction, proliferation and migration has also been found to be highly expressed in RA [24]. The mutated form of this antibody was recently developed and it is known as anti-MCV antibody. The sensitivity of which is comparable to RF but no greater specificity [45, 46]. These antibodies alone or in combination with IL-6 have a high classification power for the establishment of RA [50]. These antibodies have recently predicted erosion. They are usually associated with high titre rheumatic factor; i.e.  $\geq 50$  IU/ml. These antibodies and the presence of acute phase proteins are reliable to predict erosive RA. Anti-nuclear antibody (ANA) is also used for the diagnosis of arthritis and can be used to differentiate different forms of inflammatory arthritis [5].

## 2.2. Emerging potential biomarkers in RA

To demonstrate efficacy and define appropriate RA patients in clinical trials, the identification of easily measured, rugged, reliable markers of disease and the effects of drugs are critical and emerging [51]. Different research works are ongoing to reveal emerging and interesting biomarkers. During biomarker identification, attention needs to be paid to the selection of the biological matrix in which the particular biomarker level will be monitored keeping in mind the feasibility of sample collection from such matrix, the stability of analyte in such matrix, assay sensitivity requirement based on the anticipated biomarker level in

such matrix as well as the relevance of the matrix to biology. Biomarkers that will be translatable from bench to bedside will have to be accessible with minimal invasive procedure. Plasma and serum are the most easily accessible body fluids. In addition to this however, there should be a link between the matrix and the originating tissue source for biomarkers measured in body fluids [52]. The synovial fluid is a good source of novel biomarkers for many arthritic diseases that involve joint inflammation [53]. However, any measure used as a biomarker has to be evaluated and validated to ensure that the laboratory test is accurate, specific, sensitive and reproducible. The emerging RA biomarkers may be used to subgroup, treat and monitor the treatment [17]. The focus here is on a group of protein biomarkers characterized by a known molecular structure or formula or heterogeneous proteins with or without posttranslational modifications and some cytokines. This does not include image measurement, cell type, count, activity, or behavioural models. Novel techniques are emerging to discover protein biomarkers of inflammatory arthritis [28, 29].

### 2.2.1. Emerging biomarkers from the serum

Emerging serum markers include circulating autoantibodies against citrullinated proteins. These have high selectivity and specificity for the early diagnosis of RA [54].

- Matrix metalloproteinase 1 (MMP-1) and tissue inhibitor of metalloproteinase 1 (TIMP-1): These are tissue destructive enzymes [21]. Murphy et al, found an association between elevated levels of MMP-1 in blood and synovial tissue of RA patients and formation of new erosion [55].
- Myeloid –related proteins/Pro-inflammatory cytokines: The innate immune system of the bone activates macrophages on recognition of invading microorganisms. This activation occurs by pathogen associated molecular patterns (PAMPs) and by reacting to tissue damage recognised as damage associated molecular patterns (DAMPs). Prominent proteins released by activated macrophages include calgranulins, myeloid related proteins MRP8 (S100A8) and MRP14 (S100A9). These S100 protein families are calcium binding proteins that induce pro-inflammatory responses in leucocytes and endothelial cells [56]. The pro-inflammatory cytokines include interleukins (IL-1 $\beta$ , IL-6, IL-15 and IL-17) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) [5, 21]. A correlation exists between IL-6 and acute phase proteins; therefore IL-6 can be used to monitor disease activity in RA. However, due to its diurnal variability (variable concentrations in the morning and in the evening), it is not a reliable marker [48]. Macrophages are stimulated by inflammatory mediators such as interleukin (IL 1), tumour necrosis factor (TNF- $\alpha$ ) or interferon (IFN) to secrete and up regulate myeloid- related proteins [56]. Serum amyloid protein A (SAA), is also an emerging biomarker of RA [57].

According to previous work from peripheral blood mononuclear cells, swiprosin 1, the ezrinmoesin binding protein EBP50, and non-muscle actin have been shown to be differentially expressed in RA. Additionally, GRP78 a glucose regulated protein and the heat shock protein HSP60 have been identified as major auto-antigens in RA., GRP78 has been suggest-

ed as an immunotherapeutic agent for the treatment of arthritis and targeting HSP60 is said to be beneficial for the treatment [5].

### *2.2.2. Emerging biomarkers from the synovial fluid*

Calprotectin, a major leucocyte protein has been found in high concentration in the synovial fluid of RA patients. It is a calcium binding pro inflammatory S100 protein (S100A8/S100A9). It is also known as MRP-8/MRP14; calgranulin A/calgranulin B or cystic fibrosis antigen [58]. S100A8 (MRP-8/calgranulin A), S100A9 (MRP-14/calgranulin B) and S100A12 (calgranulin C) proteins have been found to be the most up-regulated proteins in the synovial fluid of RA patients. Although present in the serum of RA Patients, these proteins are predominant in their synovial fluid. RA can be diagnosed early by the serum expression of these proteins [24, 59]. In the synovial fluid and the synovial membrane of mononuclear cells, cytokines such as the IL-18 have been found to be highly expressed and it has also been found to contribute to inducing high levels of other monocytes such as the IL-6, TNF- $\alpha$ , IL-1 $\beta$  and the granulocyte-macrophage colony-stimulating factor (GM-CSF) [60]. Pro-inflammatory cytokines such as the IL-1 and TNF- $\alpha$  protein are also readily detectable in the synovial fluid. Other detected cytokines include the macrophage colony stimulating factor (M-CSF), leucocyte inhibitory factor (LIF), IL-6 and interferon  $\alpha$  (IFN $\alpha$ ) [26]. Studies on citrullinated proteins and autoantibodies in RA synovial fluid are ongoing and emerging; an example is the fibrinogen-derived endogenous citrullinated peptides [61].

### *2.2.3. Emerging biomarkers from the bone and cartilage*

Abnormal and degraded cartilage in affected joint is one of the major clinical manifestations in RA. Collagens are markers of bone turnover/resorption. The synthesis and degradation products of metabolism of cartilage specific collagens and proteoglycans are released into the synovial fluid, serum and urine as by products. These biomarkers can be used to monitor the metabolism of the cartilage. The main collagen of the articular cartilage is the type 11 collagen (C11) and it is a major structural component of the tissue. It is excessively degraded in RA [60]. Cartilage oligomeric matrix protein (COMP) is another marker that has been shown to provide a measure of elevated cartilage degradation [62]. So pyridinoline and deoxypyridinolin (PYD, DPD) are also specific markers of bone resorption; these are cross-linking amino acids that strengthen collagen fibrils in the extracellular matrix. PYD and DPD are found in main fibril – forming collagen 1, 11 & 111 of many tissues [63].

### *2.2.4. Emerging biomarkers from the synovial membrane /tissue*

In RA, the synovial membrane of the affected joint is the most affected part and therefore it is the primary site of inflammation. The number of macrophages in tissue biopsies of RA patients has been identified to correlate with the degree of pain. A study into Tcell infiltration within the synovial membrane during the disease has shown that there is a correlation between an improvement in the clinical index of the disease activity and a decrease in Tcell infiltration. The clinical course of RA and the response to treatments has been found to correlate with the number of sublining macrophages in the tissue. However, the synovial mem-

brane presently does not have a reliable marker for early detection of arthritis but useful for determining the prognosis of the disease [13]. The synovial biopsies from the synovial tissue are useful for diagnosis purposes as well as the evaluation of novel treatments [64]. Small chemoattractant cytokines known as chemokines play a role at the site of inflammation to accumulate inflammatory cells. The synovial tissue and fluid exhibits an increased concentration of some chemokines which includes the monocyte chemoattractant protein-4 (MCP-4/CCL13), the monokine induced by interferon- $\gamma$  (Mig/CXCL9), pulmonary and activation-regulated chemokine (PARC/CCL18), the monocyte chemotactic protein 1 (MCP-1/CCL2), the stromal cell-derived factor-1 (SDF-1/CXCL12) and fractalkine (CXC3CL1). These chemokines and their receptors are important in the pathology of RA [22]. Studies on the synovial tissue are ongoing to discover better protein biomarkers from the synovial tissue. Although not yet validated for use in clinical practice, some proteins from the synovial tissue such as fibrinogen, annexin, fibronectin, vimentin, haptoglobin, S100A8, S100A10 and some others are under study for implications in RA. Proteome analysis of the synovial tissue is promising to give further understanding on the pathogenesis of joint diseases [65].

Although all the biomarkers listed above are good indicators of RA, factors such as the presence of other diseases like osteoporosis, some variations in gene composition and tissue content of some of the biomarkers as well as increased physical activities have been found to change biomarker concentration significantly [12]. Other factors affecting marker level in mediums includes the diurnal and day-to-day activity; the level of some markers has been shown to be higher in the morning compared to the evenings. It has been found that markers are more abundant in serum samples taken early in the morning before breakfast than the samples taken after eating. Variation has also been found to occur due to eating and calcium intake assay precision has also been affected by handling, collecting and storing samples/specimens inappropriately [63]. Better and novel biomarkers are being discovered using proteomic techniques and these are described in section 3. There are a number of ways that biomarker measurements can aid in the development and evaluation of novel treatments. Biomarkers provide information for dosing and minimize differences in inter individual response to treatment as the assessment of benefit and risk is the goal of developing all therapeutic interventions [39]. It has been suggested that proteins, being 'surrogates' for the dynamic biology in organisms, are the macromolecules of choice for biofluid biomarkers [51]. Hence, a range of proteomics techniques have been applied to the discovery of novel candidate protein biomarkers [66].

### 3. Proteins and proteomics techniques in biomarker discovery

Proteomics is the study of protein expression, structure and function directed towards the characterization of the entire protein complement of a cell, tissue or organism [67]. Proteome analysis supports the determination of protein expression levels and hence monitors cellular processes [11]. Protein expression analysis in biological samples is of utmost importance to identify and monitor biomarkers for the progression of RA and its therapeutic endpoint as well as providing insight into mechanisms of the disease [68]. Differentially expressed pro-

teins and potential biomarkers of RA can be discovered using various proteomic techniques and has been applied to investigation the dynamic proteome of autoimmune diseases [66, 67, 69]. Once potential protein biomarkers have been identified, their development into diagnostic (or other) tests of clinical usefulness require significant effort and few if any biomarkers of clinical utility have emerged from proteomics. Reasons include significant limitations in the proteomics technologies used for biomarker discovery and challenges faced in their subsequent validation [70, 71].

One of the major issues to have emerged is the realisation that single protein biomarkers are unlikely to yield sufficient sensitivity and specificity. However, whilst there is much talk of multiplexed panels of markers, the application of appropriate statistical tests to the development of such a panel remains relatively poorly understood and applied. Furthermore, whilst much discovery of biomarkers has been undertaken on tissues and cells, an effective diagnostic assay may require measurement in a readily accessible patient sample such as serum or synovial fluid. Another major bottleneck has rested in the lack of opportunities or capabilities to continue the process of biomarker development to progressing them to clinical utility – a domain of translational research. The biomarker development is the step between biomarker discovery and confirmation [51]. Different strategies have been used in proteomics to identify various biomarkers of diseases. These proteomic strategies involve separation, analysis and detection of complex protein mixtures.

Historically gel-based proteomic techniques were the tool of choice to resolve complex protein mixtures followed by mass spectrometry (MS) to detect differences in protein expression patterns between normal and diseased samples. While gel-based proteomic techniques have lost favor within proteomic research groups due to its limitations, MS has remained at the forefront of proteomic biomarker discovery experiments coupled to more gel-free techniques.

### **3.1. Gel based proteomics**

Pre-separation of target proteins is highly essential in proteomics and many proteomic techniques accomplishes this with the aid of one- or two-dimensional electrophoresis [72].

One-dimensional sodium dodecyl polyacrlamide gel electrophoresis (1D-SDS PAGE) is a gel-based method that involves separating proteins on a 1D- SDS Polyacrylamide gel where by the proteins are separated solely based on molecular weight. The bands of the gel are excised and subjected to proteolytic digestion ready for analysis by MS. Identified proteins are used to provide a nonredundant list and the data from samples run in different gel lanes are compared. This is a good and powerful method for small or medium sized biomarker discovery studies [72].

Two-dimensional gel electrophoresis (2-DE) is used to separate complex protein mixtures based on their isoelectric points (pI) and molecular weights. 2-DE is one of the most powerful techniques for separating entire proteomes and was developed in 1975 by O'Farrell [73] and since has been developed further [74]. In the first dimension called isoelectric focusing (IEF), proteins are separated based on their (pI). Proteins are amphoteric

substances and therefore can either be negatively or positively charged depending on the pH of their environment. During IEF proteins are separated along thin strips of polyacrylamide gel containing an immobilized pH gradient (IPG). As an electric current is applied to the IPG strip during IEF, proteins move through the strip until they reach their pI i.e. where they no longer have a net charge. In the second dimension the IPG strip is placed horizontally along the top of a large polyacrylamide gel and proteins are separated based on molecular weight whereby smaller proteins will move faster through the gel than larger ones [51, 67]. Proteins separated by 2-DE can then be visualized by a number of staining techniques. The individual gel 'spots' may be excised from the gel, digested with proteases and the resulting peptides analysed by MS [51]. However, accuracy and reproducibility are concerns in this type of experiments [75].

Two-dimensional in gel electrophoresis (2D-DIGE) is an improvement in the use of gel-based methods for protein quantitation and detection. It has the ability to co-detect several samples on the same 2DE gel, hence eliminating gel-to-gel variation [75]. In 2D-DIGE experiments, ester cyanine dyes are used to label proteins prior to 2-DE. The advantage of using these dyes is that they are size and charge matched and so ensure negligible shift during first and second dimensions. Each cyanine dye has different excitation emission spectra allowing different samples to be run within the same gel and allows the inclusion of an internal standard on each gel. 2-DIGE alleviates the pattern reproducibility problem but not the other problems associated with 2-DE [75].

In general, biomarker discovery experiments using gel-based methods have been difficult due to the inherent limitations of the methodologies. Firstly, the hydrophobic, insoluble nature of membrane and membrane associated proteins make them incompatible with the aqueous nature of the second dimension in 2-DE and so are significantly underrepresented in gel-based studies [51]. Invariably, due to the low dynamic range of the gel-method, the most abundant soluble proteins are best represented and detected in 2-DE studies. Even with advances in IEF and staining technologies 2-DE gels are notoriously difficult to reproduce [75].

Today, proteomic studies have moved away from gel-based techniques and now use gel-free proteomic techniques.

### **3.2. Mass spectrometry-based proteomics (gel free proteomics)**

MS-based proteomics has been used for the global analysis of protein composition, modifications and dynamics and this involves three core experimental steps; (i) protein extraction which can be followed by sample fractionation (ii) enzymatic digestion and (iii) quantitative and qualitative analysis using MS [76]. For the analysis of complex protein mixtures, two MS based approaches are used; functional proteomics and expression proteomics. Functional proteomics also known as the top-down approach involves maintaining the native structure of the protein and gaining functional information on the protein. However, a major disadvantage of intact protein analysis is that it does not directly provide a sequence-based identification as there are a number of proteins with close given masses. Expression proteomics also known as the bottom-up approach involves the dena-

turation of proteins, and using subsequent MS analysis of resulting peptides to determine quantitative changes in the abundance of proteins under different conditions [77].

By definition, a MS consists of an ion source, a mass analyser that measures the mass-to-charge ratio ( $m/z$ ) of the ionized analytes and a detector that registers the number of ions at each  $m/z$  value [77]. The primary methods of ionization in MS are electro-spray ionisation (ESI), matrix-assisted laser desorption/ionisation (MALDI) or the surface-enhanced laser desorption ionisation (SELDI). Mass analysers could be the SELDI time-of-flight (often used for intact/whole protein analysis), MALDI time-of-flight, multiple stage quadrupole-time-of-flight or the quadrupole ion trap (often used for sequence-based identification) [58]. MS has solved the problem of identifying proteins resolved by 2D gel and other methods and has also been used to successfully analyse complex protein mixtures [58]. Data from Gel LC/MS often correlates with the data generated during protein assay development with the multiple reaction monitoring method (MRM) [51]. The main decision when carrying out gel-free based methods is whether to label proteins or not.

Protein labeling methods include; (i) SILAC-stable isotope labeling with amino acids in cell culture for metabolically labeled protein studies. In this technique, non-radioactive heavy isotopic forms of the amino acids are metabolically incorporated into the cellular proteins while cells are growing allowing the identification of cell surface proteins by MS [2], (ii) ICAT-isotope coded affinity tag is used to label proteins after extraction from biological samples, (iii) iTRAQ-isotope tags for relative and absolute quantitation and (iv) TMT- tandem mass tag for studies involving peptides derived from proteolytically digested biosamples. Different peptides resulting from different samples are labeled with different tags or no tags. This allows different peptides from different samples to be mixed together for mass spectrometry assay. This proteomic technique is good for studies involving small number of samples up to eight that can be easily mixed together for analysis [51].

Label-free detection methods for biomarker discovery are simpler and faster [2]. Examples include label-free mass spectrometry (MS) and multi-dimensional liquid chromatography-Mass spectrometry (LC-MS). LC-MS is an analytical proteomic technique that measures the mass-to-charge ( $m/z$ ) of peptide ions based on their motion in an electric or magnetic field. This technique is used to identify, characterize and quantify proteins based on the mono-isotopic mass of a peptide rather than the average mass of a peptide [67]. Quantitation is achieved by aligning the LC-MS data and carrying out statistical analysis across the samples. Differentially expressed peptides/protein are further analysed by MS for identification [51]. This method is good for large biosamples. Protein identification is dependent on the quality and quantity of fractionation. For more than two decades, reverse phase chromatography has been successfully used for peptide separations and it plays a key role in protein characterisation and identification. Routinely, peptide separation coupled on-line to tandem mass spectrometry equipped with electro-spray ionisation has been used for peptide sequence analysis; this has been application and sample dependent [67]. Label free proteomics is a good method as it focuses on dif-

ferential expression of proteins across groups. However, it has long time lines [51]. Examples of mass spectrometry techniques used to achieve this include the nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) along with other techniques that involve immunocapture platforms of reverse phase protein assays [30].

### 3.3. Overview of RA biomarker discovery study using mass spectrometry

The main objective of a protein biomarker discovery study is to identify proteins whose levels are significantly altered in response to some state or conditions such as treatment, disease state, mutation, etc.[51]. This technique has also been found to give insight to different signaling pathways, has improved the discovery of new therapeutic targets, and has been used to indicate response to and the duration of treatment. The three major steps in the study of protein biomarkers are discovery, assay development and validation (testing) [51]. A biomarker discovery experiment produces a list of candidate biomarker; the presence and level of which must be eventually verified in the samples [78].

In RA, biomarker discovery requires identification and quantitation of proteins in the sera, synovial fluid/tissue of RA patients. These proteins exhibit diverse physico-chemical properties [79]. The biomarker discovery in RA using MS is promising to reveal biomarkers capable of predicting and/monitor disease activity, joint damage and therapeutic response in order to minimize expense and toxicity [17]. To accomplish this, early and better prognostic markers are required[13]. The steps involved in protein biomarker discovery experiment using proteomic techniques are as highlighted below.

- Sample collection and storage -This is the most crucial step in proteomics study. Quality samples are difficult to obtain and there are no means to test the quality of proteins in different samples. Suggestions include limiting the time of exposure of samples to room temperature, keeping samples frozen at -80°C as changes in protein are known to occur very quickly. Collection of serum or plasma from patients should also be carried out following standard operating procedures (SOP) for plasma collection [51].
- Sample preparation -This is the second crucial step in sample analysis. Sample preparation involves the disruption of cellular matrix as homogeneity of samples is essential; solubilization of proteins for example in detergents; fractionation of the complex protein mixture due to the diverse abundance of proteins in the mixture, depleting the most abundant proteins-albumin, IgG; protein digestion into peptides for MS analysis using trypsin; removal of nonprotein/nonpeptide molecules for some sample types such as the synovial fluid and urine. Methods of removing the interfering molecules include electrophoresis through polyacrylamide gel or solid phase extraction (SPE) [51].
- Sample assay -MS has been successfully used to analyse the differential patterns of protein and peptide expression in patient biospecimen. This is a high throughput approach used to assay for millions of peptides [30, 69]. Protein quantitation can be achieved by relative or absolute quantitation either by measuring the chromatogram peak area or spectral counting [51]. Potential biomarkers can be identified from differentially expressed

proteins among different groups of samples from a proteomic discovery experiment with the aid of statistical analysis. The acquisition of knowledge on the function of the discovered potential biomarkers is the main goal of all proteomic research. This is achieved with the aid of external databases and the literature to know the involvement of each protein biomarker in different pathways and processes depending on the location of the protein in the cell [67].

- Statistical analysis - Different software tools are available for differential analysis of proteomic data. Usually the differential analysis between diseased/healthy materials, mutants/wild type species, treated/control samples is the best approach to study changes in protein expression levels. However, studies have shown that it is rare that proteins are either absent or present but they are in most cases up or down regulated in different samples. Hence, there is a need for a precise and confident analysis of the quantitative changes [67]. The type and amount of statistical analysis depend on the number of biological and technical replicates. Technical averages and variances are used to calculate the biological averages and variances. Analytical and technical variability and CV (coefficient of variation) should ideally be less than 10% while biological variation may be high [51]. Across groups, fold change calculations are done to identify differentially expressed proteins. Although, arbitrary cut-offs are used looking at the data and this is usually 1.5- or 2- folds change. For three or more biological replicates, pvalue (t-test) and/or false discovery rates (FDR) are calculated for the data analyses with cut-offs typically  $p < 0.05$  and  $FDR < 20\%$  considered significant respectively. Multivariate analysis such as hierarchical clustering, principal component analysis and other statistical programs are used for data analysis [51].

### 3.4. Verification and validation of protein biomarkers in RA

After the discovery phase of biomarkers using proteomic techniques, there is a need to confirm the biomarkers [51]. Verification is the paradigm shift from unbiased discovery experiments to targeted, hypothesis-driven methods [78]. It is necessary to reliably identify and reproducibly quantify a potential protein biomarker of interest over multiple samples before establishing its value as a protein biomarker [68]. There is a need to prove the functions of the potential biomarkers discovered by a second entirely independent analysis method. Western blotting and multiple reaction monitoring (MRM) methods are often used for this task [67]. LC-MS/MS using MRM is gaining acceptance as the primary analytical tool for quantitation of small molecule biomarkers in biological fluids. This is the quantitation of proteins using proteolytic digestion followed by MRM quantitation of unique peptides to the protein of interest [52]. Peptides with analytical or technical variance  $> 20\%$  are not suitable for a multiple reaction monitoring (MRM) assay [51]. A complete set of analytical samples with quality controls and standards are used for validation [52].

Analytical or technical validation is known as verification and this process confirms the assay performance characteristics as well as the required optimal conditions that will give reproducible and accurate data. The behaviour of a marker within and between populations gives the clinical and biological validation [30].

The procedures taking place after the development and optimization of bioanalytical procedures is known as validation. The word validation is broad and has been described as the process of linking a biomarker to clinical or behavioral endpoints [30]. These are aimed to show that the method is “fit for purpose”; and that the procedure is reproducible and reliable for its intended use. The fundamental parameters involved include accuracy, selectivity, sensitivity, stability, reproducibility, precision as well as effectiveness of results. [52, 55]. This process of reproducibly quantifying multiple proteins in complex backgrounds over large cohort of patients’ specimen is highly important in biomarker research [80]. Without verification and validation of biomarkers, they cannot be used as drug response marker(s) in clinical practice [30].

There are two common types of assays developed to verify and validate the proteins of interest. These are the antibody-based assays and the MS-based assays. Both approaches can be complementary [51]. To develop the protein biomarkers discovered are some proteomic methods discussed below:

1. Antibody based assays - autoantigen microarrays for the characterisation of antibody responses, reverse phase protein array studies to analyze phosphoproteins, antibody array technologies to profile cytokines and other biomolecules (e.g western immunoblot assay, enzyme-linked immunosorbent assay –ELISA, platform specific assays such as luminex); flow cytometric analysis of phosphoproteins. However, each has their limitations. Antibody-based assays are highly sensitive, has medium to high specificity, low multiplexing capability (1-10 proteins), assay development is time consuming and expensive, and it has a low success rate as it is difficult to find a pair of antibodies with high specificity [51, 79, 80].
2. Mass spectrometry based assays- Peptide multiple reaction monitoring (MRM) assays have emerged as an alternative to affinity-based measurement of proteins [80]. This is a targeted proteomic method where the mass spectrometer is directed to monitor specific peptides for the proteins (biomarkers) of interest. This method involves a protein list and sample type for the assay, selection of unique peptides to the proteins of interest, selection of fragment ions of the peptide detectable by the mass spectrometer, tuning the mass spectrometer to look for the peptides and fragment ions in the samples (first for individual peptides and then multiplexed for multiple peptide/fragments ions for each protein), testing and selection of best peptides for the assay, as well as checking for technical, analytical and biological variability using different samples [51]. Quantitation is achieved in MRM assays from the peak areas of the fragment ions for each peptide. Results are refined to get the final list of peptides and product ions. The refined final list can then be used to test samples for absolute or relative quantitation comparing values across samples. For relative quantitation, a sample is chosen as the reference to which other samples are compared while absolute quantitation of protein concentration involves labeled and unlabeled standards for each peptides; calibration curves constructed from labeled peptide standards are used for peptide quantitation [51]. MRM has a high accuracy, high throughput, supports lower detection limits for peptides and supports the measurement of multiple

proteotypic peptides and efforts are ongoing to improve the development of MRM assay softwares, precision, accuracy and robustness [80]. MS based assays have a medium sensitivity, high specificity, high multiplexing capability (ability to quantify multiple proteins in parallel), takes a shorter period of time to develop assays compared to the antibody-based assays, has a high success rate and it is not as expensive as the antibody-based assay (cost- efficiency). It is highly reproducible across different instrument platforms and laboratories and has the potential to bridge the gap between generating candidate list and their clinical use [51, 80].

It has been predicted that the use of MRM protein assay will increase the number of validated medically important protein biomarkers. MRM can provide both relative and absolute quantitation of peptides like the antibody-based assay. MRM assays has three major advantages over the antibody based assay i) High specificity for the protein of interest or its isoform; ii) short time lines for assay development and iii) high multiplexing of the assay to include 25 proteins or more in a single assay [51].

The detection level of proteins using the MRM method depends greatly on the detection of the protein in a previous biomarker discovery experiment using the GeLC/MS or label free LC/MS/MS. Other proteins selected as potential biomarkers based on literature review or other experiments such as transcriptomics may likely exist in low abundance in the samples of interest. If need be, these protein can be boosted using different enrichment strategies[51].

Important to the development of a successful MRM protein assay is the detection of fragment ions that are well separated and sufficient data points obtained for each peak. In a typical MRM assay, the separation of peptides are based on the retention time in the liquid chromatography (LC) as well as their mass/charge ( $m/z$ ) while the peak areas for the fragment ions for each peptide is used for the quantitative analysis [51].

Novel biomarkers discovered, verified and validated with proteomics are critical in the development of targeted compounds thereby directing rational treatment to patients. In many autoimmune diseases, studies are underway to define the inflammatory proteome, disease proteome, vascular proteome and other subsets of the pathologic environment. Potential biomarkers when verified and biologically validated are promising to lead to the selection of individuals most likely to benefit from treatment. MRM protein assay developed may be used routinely for testing biological samples [30, 51].

Using different proteomic techniques, a number of potential RA protein biomarkers are under study. These includes serum amyloid A [57]; S100 family of calcium binding proteins found to regulate joint inflammation and cartilage in arthritis [56] and many more as listed in Table 2. None of the available proteomic methods has emerged as the best for all proteins biomarker discovery studies. Each method has their pros and cons. The success of proteomic studies depend on sample quality, the technical variability of the method as well as the depth of protein analysis. The best platform for proteomic studies depends on factors such as the number of available samples, the timeline for completion of study and the funds available for the study [51].

Sample type	IA associated proteins	Sample preparation	Mass Spectrometry platform	References
Plasma	Actin, CRP, Calgranulin A, B and C (S100 family of calcium binding proteins – A4, A8, A9, A11, A12 and P)	Immunodepletion, GC and LC separation	2-dimensional LC/LC and MS/MS; ESI Triple Q/MRM	[29] [65]
Serum	Serotransferrin, Serum amyloid A, GAPDH, Alpha-1-antitrypsin, Citrullinated fibrinogen, Apolipoprotein A11, Vitamin D binding protein, C-reactive protein, S100A8, S100A9, S100A12 and $\alpha$ -defensins	Acetone precipitation, 2DE, CIC/DIGE Immunodepletion +size exclusion chromatography (SEC) Immunoprecipitation +size exclusion chromatography (SEC)	LC MALDI TOF/TOF LC/ESI-MS/MS MALDI TOF-TOF LC ESI Triple Q/MRM SELDI-TOF-MS	SEE [81] [82] [83] [53] [84] [85] [57]
Synovial fluid	C-reactive protein, S100A8, S100A9, S100A12, S100A4, S100P, S100A11, Apolipoprotein A1, Cathepsin B, Peptidyl prolyl isomerase, Triose phosphate isomerase, 14-3-3-protein alpha_beta, Osteopontin, Transgelin 2, Kininogen, Vitamin K-dependent protein C, $\alpha$ -defensins, Citrullinated fibrinogen, Calgranulin A, C (MRP 14 and MRP12)	Immunoprecipitation, Immunodepletion +size exclusion chromatography (SEC) Reverse HPLC fractionation Ultracentrifugation	LC ESI Q/TOF HPLC/LCQ ion trap LC MALDI TOF/TOF SELDI-TOF-MS LTQ-FT-ICR	[29] [85] [61] SEE [65] [29]

Sample type	IA associated proteins	Sample preparation	Mass Spectrometry platform	References
Synovial Tissue	Aldolase A, Annexin,	SCX, acetone	LC ESI ion trap/SRM	[29]
	Calcium-binding	precipitation	MALDI-TOF MS	[86]
	S100 proteins,			[87]
	Cathepsin D, CRP,			[88]
	ENOA, Ig κ-chain, MnSOD, NGAL, PRDX2, PRDX4, SOD2, TG2, TXNDC5			
Urine	Transferrin, Serum amyloid A,	1D gel, 2DE, Immunoprecipitation,	LC MALDI Triple Q/MRM	SEE [81]
Pannus tissue lysate	Citrullinated fibrinogen	1D GEL/immunoprecipitation	LC ESI ion trap	[83]
Whole saliva	14-3-3 protein, apolipoprotein A, calgranulin A and B, E-FABP, GRP78/BiP, PRDX5	2D-DIGE	LC/MS/MS	[89]

E-FABP=Epidermal fatty acid binding protein, PRDX=Peroxiredoxin; GRP78/BiP=Glucose related protein precursor; TXNDC5=Thioredoxin domain-containing protein 5; MnSOD=Manganese superoxide dismutase; MRP = Myeloid related protein; CRP= C-reactive protein; SOD= Superoxide dismutase, GAPDH= Glyceraldehyde 3-phosphate dehydrogenase, ENOA= enolase, NGAL= d neutrophil gelatinase-associated lipocalin TG2= Tissue transglutaminase 2.

**Table 2.** A review on inflammatory arthritis associated proteins and mass spectrometry techniques used for identification. IEF=Ion exchange chromatography; 2D-DIGE=Two dimensional- differential in gel electrophoresis; ESI-MS=Electrospray ionisation mass spectrometry; 2-DE=Two dimensional gel electrophoresis; MALDI-TOF-MS=Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry; SELDI-TOF-MS=Surface-enhanced laser desorption ionisation time-of-flight mass spectrometry; IP=Immunoprecipitation; ID=Immunodepletion; SEC=Size exclusion chromatography; LC=Liquid chromatography; HPLC=High performance liquid chromatography; MRM=Multiple reaction monitoring; LTQ-FT-ICR=Ion trap-Fourier Transform mass spectrometer.

### 3.5. Clinical utility of protein biomarkers in RA

The ultimate aim of RA treatment is to achieve and sustain remission but current targets include the suppression of disease activity, the improvement of functional ability and the slowing of joint damage [90]. Accurate measurement of disease activity can be used in therapy management as it should guide in ensuring that effective therapies are continued and ineffective ones discontinued [31]. Treatment response in RA is a measure of the suppression of inflammation solely with acute phase response indicators - CRP and ESR or in combination with clinical information. Prognostic factors that may affect response or no response to treatment include the presence of rheumatoid factor, rheuma-

toid nodules, HLA-DR4/sharp epitope and anti-cyclic citrullinated peptide antibodies although these cannot be used to predict response to therapy. Hence, there is a need for new biomarkers to predict response to therapy and to help in preventing long-term radiographic progression in patients [91]. Validation of new multiplex assay and technologies are essential before clinical applications [52].

A number of factors are responsible for the inability of lots of potential protein biomarkers reaching clinical utility. These factors have made the translation of biomarkers from bench to bedside difficult [51]. Decision making is one of the main factors for the utility of biomarkers hence, there is a need for all stakeholders in the decision to be involved in the biomarker development. Therefore, in addition to biologists, pharmacologists, medical practitioners in the appropriate fields, analytical experts are needed for the identification and quantification of potential biomarkers. They should all be involved in the analyses and optimal use of the data [92, 93]. With different biomarkers at different stages of validation, clinicians and researchers are finding it difficult to make a sense of it all [80].

### **3.6. Challenges /future of biomarkers and proteomics for inflammatory arthritis**

Proteins are difficult molecules to monitor with the first technical issue being the insufficient depth of the current methods in regards to the broad range (12 orders of magnitude) of protein concentrations in biofluids. There are different orders of magnitudes for the dynamic range of most methods [51]. Another major limitation in translational research particularly in the validation step of protein biomarker include lack of reproducible, accurate and sensitive assays for most potential biomarker proteins described in the literature. However, MRM has been reported to allow reproducible protein quantification [80]. Technical variability is a key factor that affects the design of experiments in proteomics. The higher the technical variability, the higher the number of technical replicates that needs to be done and this is evaluated by comparing data from the replicates of the same sample. Comparing the data from the replicate samples, the correlation coefficient ( $R^2$ ) calculated gives an indication on the technical variability.  $R^2 > 0.9$  is an indicator of low technical variability,  $R^2$  between 0.8 and 0.9 is acceptable and  $R^2 < 0.8$  is an indicator of high technical variability and a biological signal might be difficult with the high technical variability [51]. Multiplexing assays whereby multiple analytes are measured from the same sample is often used to fully understand the correlation between the biomarkers and the underlying biological pathways or to investigate the multiple potential biomarkers before deciding on the decision-making biomarker. Variability that may occur due to limited availability of samples or different separation methods can be minimized by multiplexing assays on an LC-MS/MS platform. Flow cytometry based technologies and planar-array technologies also have multiplexing options. There is a need to validate these new multiplex assay platforms before recommending them for clinical use [52]. In addition, most proteomic techniques are highly sophisticated to operate and have high demand for hands-on skills. Good operator knowledge and skill as well as the performance of the instrument are equally important. Therefore educative programs and tutorial are indispensable in proteomic societies [67].

The effect of combined therapy on response as well as the knowledge of predictive biomarkers of response is a potential emerging area in inflammatory arthritis, as this will promote personalised medicine. Future studies should aim at the knowledge and better understanding of the changes occurring in the synovial tissue prior to and upon administration of anti-TNF- $\alpha$ . In addition, further studies on the identification of biomarkers that are down regulated by TNF- $\alpha$  inhibitors can be another useful therapeutic target.

#### 4. Conclusions

The pace of discovery and development of protein biomarkers of IA is accelerating with the use of a range of proteomic techniques. Together, these have the opportunity to make a significant impact on the treatment of IA. However, the challenges associated with realising this potential as well as the progression of new biomarkers to clinical utility are significant.

Targeted therapy through the emerging proteomic technologies will help select patients who may be more likely to benefit from personalised medicine and this may bring about the clinical adoption of molecular proteomic stratification. Comprehensive proteomic profiling and trial-focused endpoint profiling will be critical for development of biomarkers and potential drug targets. Proteomics will also aid the understanding of the potential protein biomarker signaling pathways to define the preferred targets of molecular therapy. The discovery and validation of new biomarker signatures will broaden our understanding of the disease and may lead to development of new potential drugs for personalised medicine in IA.

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