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# **Opportunities and challenges for the discovery and validation of proteomic biomarkers for common arthritic diseases**

Khadija Ourradi PhD & Mohammed Sharif\* PhD

**Running Head:** Discovery and validation of OA and RA biomarkers

School of Clinical Sciences, University of Bristol, Musculoskeletal Research Unit, Learning and Research Building, Southmead Hospital, Bristol BS10 5NB, UK.

([Khadija.ourradi@bristol.ac.uk](mailto:Khadija.ourradi@bristol.ac.uk), [mo.sharif@bristol.ac.uk](mailto:mo.sharif@bristol.ac.uk))

\*To whom correspondence should be addressed. Dr M. Sharif, School of Clinical Sciences, University of Bristol, Musculoskeletal Research Unit, Learning and Research Building Level 2, Southmead Hospital, BS10 5NB Bristol, United Kingdom. +44 117 414 7926, Email:

[mo.sharif@bristol.ac.uk](mailto:mo.sharif@bristol.ac.uk).

## **Authors' contribution:**

KO and MS collected data, prepared the manuscript. All authors read and approved the manuscript.

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**Abstract**

Osteoarthritis (OA) and rheumatoid arthritis (RA) are the most prevalent of all the rheumatic diseases and currently, there are no reliable biochemical measures for early diagnosis or predicting who is likely to progress. Early diagnosis is important for making decisions on treatment options and for better management of patients. This narrative review highlights the first-generation biomarkers identified over the last 2 decades and focuses on the discovery and validation of candidate OA biomarkers from recent mass spectrometry-based proteomic studies for diagnosis and monitoring disease outcomes in human. It discusses the challenges and opportunities for discovery of novel biomarkers, progress in the development of techniques for measuring biomarkers, and provide directions for future discovery and validation of biomarkers for OA and RA.

**Keywords:** Biomarkers, proteomics, mass spectrometry, osteoarthritis, rheumatoid arthritis.

## **Introduction**

Arthritis is one of the most common causes of pain and disability in the community, affecting people of all ages and gender although it is more common in women than man. Arthritis is a major drain on the economy due to loss of productivity and absence in the work place, medical and non-medical costs and costs incurred due to poor quality of life. Many of these costs adversely affect the patients and their families [1-3] and therefore arthritis is a major socioeconomic problem today. There are approximately 200 different arthritides of which osteoarthritis (OA) and rheumatoid arthritis (RA) are the most prevalent. In this review, we focus on the discovery of OA biomarkers from recent proteomic studies and discuss the opportunities and challenges associated with mass spectrometry (MS)-based identification of proteomic biomarkers and their development into suitable point of care diagnostic tests. We draw on our recent experiences of discovery and validation of OA biomarkers and provide directions for future research in this field.

## **Pathophysiology of OA**

OA is a disease of the synovial joint organ where patient clinically present with symptoms such as chronic joint pain, tenderness, stiffness and crepitus (cracking) of joints with movement [4]. It is the most common chronic joint disease [5] and is becoming increasingly prevalent as the population ages. Obesity is a major risk factor for developing OA and recent data suggests that there will be an epidemic of obesity-related OA in the general population in the future [6]. By 2020, OA will be the fourth leading cause of disability in the world [7]. It is estimated that in UK radiographic evidence of OA is present in as many as 80% of the people who are in their late 50s [8]. The common joint sites affected by OA include knee, hip, hand, spine and big toes. The risk factors for developing OA at different joint site are very different but the processes of joint damage following the initial trigger is similar at all joint sites. Knee OA is the most common and two regions of the knee joint are usually affected, the tibiofemoral (TFJ) and patellofemoral (PFJ) areas, although the TFJ compartment is commonly affected first. OA pathology is characterised by focal loss of cartilage, especially in early stages of the disease, osteophytes, sclerosis, variable inflammation of the synovium and the presence of cysts [9]. The progressive loss of articular cartilage (AC), involves degradation of articular cartilage, alongside attempted repair [10].

The American College of Rheumatology (ACR) has developed diagnostic criteria for OA at various joint sites [11-13] based on the original diagnostic criteria developed by Kellgren and Lawrance [14]. OA develops over many years and can be asymptomatic and is active at sub-clinical level long before a diagnosis can be made. Therefore, new and more sensitive tools are required for studies of OA and many joint tissue-specific molecules have been investigated as potential markers of the disease process(es) in OA.

### **Pathophysiology of RA**

RA is a systemic chronic inflammatory disease where patient clinically present with symptoms such as tenderness, swollen and morning stiffness of the affected joints. It is typically characterised by bone resorption, inflammation of the synovium and cartilage loss [15]. RA causes progressive destruction of cartilage and bone with radiographic joint erosion being the most important outcome measure [16, 17]. The aetiology of RA is unknown but historic and recent studies suggest that both genetic (particularly HLA-DRB1) and environmental (smoking, lifestyle, socioeconomic status etc.) factors are involved in the pathophysiology of RA [18-20]. Interestingly, a small percentage of patients with RA enter remission after about two years, and approximately 20% develop chronic progressive disease despite receiving treatment [21].

The presence of inflammatory cells and pro-inflammatory cytokines in synovial fluid (SF), and circulatory auto-antibodies such as rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs) are also hallmarks of RA. RFs and ACPAs are auto-antibodies against self-IgG and citrullinated peptides respectively and their presence correlate with more severe disease progression and a higher mortality in RA patients [22, 23]. Recent studies have also identified epigenetic events (including DNA methylation, histone modification, and microRNA expression) and microbiome associated risk factors for development of RA. For an excellent review of these risk factors please see the article by Firestein *et al* [24]. RA often leads to extra-articular risks such as increased cardiovascular disease [25] leading to functional impairment, long-term disability and mortality [26, 27].

In 1987, the ACR developed the RA classification criteria partly to help maximise homogeneous populations for clinical trials [28]. However, the sensitivity and specificity of the criteria in early RA were 40-60% and 80-90% respectively and therefore unsuitable for diagnosis of early RA [29]. The

diagnosis of early RA depends on the onset of symptoms and can only really be given by the patients themselves which can be inaccurate, biased and therefore unreliable [30]. In 2010, the ACR and the European League Against Rheumatism (EULAR) suggested a new classification criteria for RA, to improve the sensitivity of detection of early RA by focusing on identifying chronic erosive disease [31]. This new classification cannot solely be used for definite diagnosis of RA but only support it as the criteria involve the scoring of both clinical and laboratory measures to establish a diagnosis of RA including the numbers of small or large joints, the length of the disease, the presence of serum autoantibodies and the increase of acute phase reactants.

Over the years, studies have suggested that the early treatment of RA is correlated with better outcomes in patients which led to the concept of “window of opportunity” [32]. This concept is now widely accepted in the scientific community and support that treatment during a specific window during the early course of the disease could alter the progression of the disease by preventing inflammation and erosion leading to a long-term improvement [32-34]. Identification of specific and reliable serological markers of RA is a crucial unmet need in the field and the availability of such biomarkers will improve patient care via early diagnosis and identification of the “window of opportunity” for more effective treatment.

### **Diagnosis of OA and RA and possible role of biomarkers**

Currently, conventional radiography is the ‘gold’ standard for diagnosis and monitoring OA however plain x-ray is rather insensitive and provides little or no information on soft tissues. Additionally, actual cartilage thickness cannot be measured directly by x-ray and so the loss of joint space width (JSW) is used as a substitute and usually by the time a definite radiographic diagnosis is made, the disease is often in advanced stages [35]. To improve detection, Magnetic Resonance Imaging (MRI) can be used early on and can be predictive of radiographic change as it can directly visualise all articular tissues [36], but may not be cost-effective. Plain x-ray is also used to assess joint erosions and loss of joint space in RA. Ultrasonography can detect synovitis and cortical bone lesions in RA [37, 38] and is increasingly used for diagnosis of RA. Several independent studies have showed that ultrasound was better than clinical examination in up to 75% of patients [39], and in 2013 ultrasonography and MRI were recommended by EULAR to help confirm the diagnosis of RA [38].

Dual-energy X-ray absorptiometry (DXA), bone mineral density (BMD) can be measured at specific regions of interest (ROI) in OA. Increased BMD is associated with an increase in Kellgren and Lawrence (KL) score, sub-chondral sclerosis and minimum JSW. DXA has its limitations whereby the position of the knee must be identical in serial readings for accurate data. Soft tissue swelling may prevent the full extension of the knee preventing the DXA image from being within the ROI. There is also an overlap in BMD readings between OA and non-OA patients using DXA [40]. Scintigraphy bone scans have been used in research for investigation and assessment of early OA and can provide crucial information on the underlying pathology of the disease process [41], however, it delivers a relatively high radiation dose. Accordingly, new more sensitive and less invasive tools are required for both early diagnosis and monitoring disease progression in OA.

There are several common pathological features in OA and RA including loss of cartilage, changes in the subchondral bone and synovium inflammation. However, the pattern of changes in each of the joint tissues in the two conditions is very different. For example, in early OA cartilage loss tends to be focal while in RA, cartilage is lost from the whole of the articulating surface of the affected joints. There is increased bone remodelling in OA while in RA there is a loss of bone, and RA is typified by marked inflammation of the synovial membrane while only mild to moderate inflammation of the synovium is usually seen in OA. These pathological changes in the joint tissues of OA and RA lead to release of joint tissue-specific biomarkers into the SF, serum and eventually urine. Therefore, the concentrations of these biomarkers in the body fluids is likely to reflect the different disease processes in OA and RA joints.

Over the last two decades, a large number of biomarkers have been identified for the investigation of OA and RA and extensive studies of biomarkers *in vitro* as well as *in vivo* (in OA and RA patients) have led to the identification of some biomarkers which are clearly useful and others of questionable value. Many of these biomarkers have been validated in our laboratory using well-characterised cohorts of patients [10, 40, 42]. For example, our early studies demonstrated that SF osteocalcin (OC) (a marker of bone formation) correlates positively with scintigraphy scan abnormalities [43] while serum OC and markers of bone degradation N-telopeptide of type I collagen (NTX) were unrelated to scintigraphy scan abnormalities [44]. Serum markers of cartilage and synovial tissue turnover (cartilage oligomeric matrix protein (COMP), cartilage glycoprotein-39

(YKL-40), hyaluronic acid (HA) and sensitive C-reactive protein (senCRP)) were higher in patients with scintiscan negative knees compared to scintiscan positive ones [44]. These studies helped to identify biomarkers for the different pathological processes (e.g. bone remodelling, cartilage loss etc.) in arthritic joints, but none of these biomarkers is sufficiently specific for use in individual patients.

Elevated levels of autoantibodies such as RFs and ACPAs are good indicators of the presence of RA. RF antibodies are not strictly specific for RA but can be detected in the serum of 70 to 80% of patients with RA and therefore are important diagnostic and prognostic marker of RA. ACPAs are the most specific biomarker for RA so far, with sensitivity and specificity of 67% and 95% respectively [45]. ACPAs appear as early as 10-14 years before the beginning of symptoms and therefore a reliable marker of disease progression in RA [46, 47]. In addition, acute phase reactants (APR) biomarkers are usually used to assess RA activity which includes Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) but their sensitivity and specificity are lower than autoantibodies [48].

Biomarkers are a non-invasive way of measuring disease activity in RA and OA, and provide a cost-effective way of investigating these diseases. For example, there are a number of commercially available biomarker assay kits for investigation of OA and RA (such as COMP, C-telopeptide of type II collagen (CTX II), HA etc.) which cost on average about £500 for 96 tests, and will do 40 samples when assayed in duplicate (£12.5/sample). These kits are much cheaper than the NHS costs for adults x-ray and MRI (without contrast) for a single knee which are £46 and £145 respectively and costs 2 to 3 times more in private sectors [49]

The more information about arthritis that health workers can glean from results of these assays, the more able they will be, in the future, to diagnose early and treat the diseases appropriately. In the long-term, this could potentially reduce the economic burden due to medical and non-medical costs, lost working hours and increase the quality of life for the patients and their families. Biomarkers can provide information about the pathology at the time of sampling [40], can be used for early detection of the disease and monitor effect of treatment. Most currently available biomarkers can be classified as markers of a particular process e.g. synthesis, degradation and turnover. However, in 2006 a classification system of OA biomarkers known as the "BIPED" (**B**urden of disease, **I**nvestigative, **P**rognostic, **E**fficacy of intervention and **D**iagnostic) was



proposed by Bauer et al [50] in order to improve our understanding and to help disseminate the results of OA biomarker studies with a common language. Despite the active research in this field, none of the first-generation biomarkers discussed above (except ACPAs) have proven to be sufficiently specific for early diagnosis, predicts the course of disease with time, or monitor response to therapy for either OA or RA. The recent advances in the proteomic technology are providing a new opportunity for discovery of better biomarkers. The omics technologies collectively have already resulted in more than 150,000 papers on biomarkers, but only about 100 biomarkers have been validated for routine clinical practice for various disorders other than arthritis [51].

### **The mass spectrometry technology**

The development of new technologies in the proteomics field have been driven by the necessity to detect and quantify the vast abundance of proteins/peptides in biological samples, providing insight into the biological and pathophysiological processes, leading to the discovery of new biomarkers for investigation of many chronic diseases. A number of different MS-based proteomic technics have become available in the last decade (see Table 1) as the primary instrument for largescale protein analysis and are constantly improving in terms of mass accuracy, resolution and sensitivity to face the analytical challenges of the rich protein range of biological samples. Proteomic discovery studies are commonly used to give a proteome overview of a given sample, in the medical context that may aim to detect/quantify the expression of proteins changes related to a different condition such as disease versus healthy states.

**Table1:** Range of MS-based proteomic techniques currently available for biomarker discovery

<b>Mass spectrometry</b>	
<b>Ionization Techniques</b>	Electrospray ionization (ESI)
	Matrix-assisted laser desorption ionization (MALDI)
	Surface-enhanced desorption ionization (SELDI)
<b>Mass analyser</b>	Linear ion trap (LIT or LT)
	Quadrupole (Q)
	Time-of-flight/time-of-flight mass spectrometry (TOF/TOF-MS)
	Fourier transform ion cyclotron mass spectrometry (FTMS)
	Ion Trap
<b>Quantification Techniques using mass spectrometry</b>	<b>Relative quantification</b>
	Isotope-coded affinity tag (ICAT)
	Non selective isotope-coded protein labelling (ICPL)
	O <sup>16</sup> / O <sup>18</sup> labelling
	Stable isotope labelling of amino acids in mammals (SILAM)
	Stable isotope labelling with amino acids in cell culture (SILAC)
	Label free quantification (LFQ) (semi quantitative approach)
	<b>Relative &amp; absolute quantification</b>
	Isobaric tags for relative and absolute quantification (iTRAQ <sup>®</sup> )
	Tandem mass tags (TMT <sup>®</sup> )
	Isobaric peptide termini labelling (IPTL)
	<b>Absolute quantification</b>
	Absolute quantification (AQUA),
	Quantification concatemer (QconCAT)
	Protein Standard Absolute Quantification (PSAQ <sup>™</sup> )

Mass spectrometers are composed of three important components: an ion source, a mass analyser, and an ion detector. Molecules are first converted into gas-phase ions (ionisation techniques). Ions are then separated in a mass analyser on the basis of mass-to-charge ratio and finally, ion strike a detector generating a record of the number of events or the electrical current created [52, 53]. In short, two strategies could be summarised for proteomic studies: analysing the full-length protein (top-down method) and analysing peptide resulting from enzymatic digestion of the proteins (bottom-up method). “Top-down” protein analysis approach allows for the measurement of whole intact protein permitting a full characterisation of the protein (such as molecular weight, protein type, location and relative abundance of post-translational modifications (PTM)) [54, 55]. It requires little time for sample preparation however, this method can be challenging due to difficulties with protein fractionation, reduced ionisation, longer MS acquisition times and detection for proteins with increasing molecular weight [56, 57].

The “Bottom-up” proteomics approach also called “shotgun” is the most commonly used MS-based method for protein analysis [53, 58, 59]. It consists of identifying proteins by analysis of peptides levels released by enzymatically or chemically cleaved into peptides. Digested peptides are separated by LC before being ionised and subject to tandem MS (MS/MS) that provides a spectrum for each peptide. Using an algorithm, acquired MS/MS spectrums can be compared to in silico proteins sequences in a database for identifications. However this method is insensitive to protein isoforms; for instance, it may not identify PTMs that occur on peptides, causing their relation to one another to be lost following digestion [52].

There are numerous mass spectrometers that can be used in proteome research [60]. The combination of MS instrument allows for a better accuracy, sensitivity and speed of analysis going beyond identification and allowing for quantification of peptides/proteins of interest. Indeed, over the years different strategies have been developed for relative quantification and absolute quantification. The former providing measurement of protein levels amongst different samples expressed in fold change of protein abundance and the later providing exact measurement of a protein using both stable isotope label and label-free approaches (Table 1). At present, there is no method or instrument that is able to identify and quantify in single-step operation the components of a complex protein sample [60].

When it comes to choosing the best MS-based approach for accurate protein quantification or biomarker discovery, one should consider the reproducibility of the assay, the representation of the protein spectrum that will be analysed (low-abundance protein) or/and the need for detection of PTM. In addition, the methods utilised prior to quantitative MS such as depletion, enrichment and fractionation should be carefully considered as they can introduce error in the measurement of the original sample.

Stable Isotope labelling consists of labelling a protein or peptides of interests metabolically or chemically with a differential mass tag that will alter its mass but not its chemical properties during chromatography or MS [61]. The principle is to use MS to compare the amounts of a labelled molecule “heavy” (known concentration) against the endogenous “light” isoforms to determine the relative or absolute quantification in a sample [61]. In contrast, the label-free approach can achieve relative quantification based on peptide peak intensity and spectral

counting. These approaches and their uses have been widely described and published in many reviews [62, 63] so we are not going into further details regarding their application but rather emphasise that any methodology used will have pros and cons and will define the type of biomarkers selected. For instance, the label-free approach as described by Wasinger *et al*, will be less consistent for low mass proteins and more reliable for higher abundant proteins as more abundant proteins will produce more MS/MS spectra. It has also been reported that relative quantification by using peak intensity measurements could be affected by condition variation such as instrument calibration and sensitivity in a long-term project and introduce up to 40% discrepancy at the peptide level between samples [62]. However, label free techniques are fast, easy and inexpensive to perform and offer an alternative to isotope labelling [62, 64]. Contemporary mass spectrometers have exceptional sensitivity, providing detection at attomole concentrations [65]. However, although MS-based proteomics allows for broad analysis of high abundant proteins it has still not overcome the challenge of analysing low abundant protein in complex biological samples.

### **3. Biomarkers from recent proteomic studies**

With the rapid recrudescence of the omics technologies, identification of disease specific biomarkers is greater than ever, and many potential new biomarkers for OA have been identified during the last 6 year (Table 2). A broad range of different biological samples, including articular cartilage from femoral heads and knee, SF, serum and urine, have been investigated in proteomic studies to inform about the differential expression and composition of molecules involved in OA disease.

**Table 2:** A list of the MS- based proteomic studies in the last 6 years comparing results of analysis of samples from OA, RA patients and control subjects. (Listed from old to new publication)

Proteins/peptides of interest	Method	Sample	Protein confirmed by WB and/or ELISA	Reference
170 targets detected with 6 identified proteins only found in healthy and 9 only in OA	LC-LTQ	Articular cartilage vesicles isolated from 10 normal and 10 OA human knee cartilages	None	[66]
4 targets detected with 2 identified as C3f and V65 showing differential expression in OA in comparison to RA and healthy control	SELDI-TOF	Sera from diagnosed OA patients, diagnosed RA patients and individuals with no inflammatory / joint disease history	None	[67]
368 targets detected with 357 relatively quantified and 38 significantly modulated when chondrocyte stimulation with IL-1 $\beta$ .	LC-MALDI-TOF/TOF	Chondrocytes isolated from articular cartilage of patients OA undergoing total joint replacement.	None	[68]
262 targets detected with 6 proteins differentially expressed in moderate OA, 13 in severe OA and 7 in both.	LC-MALDI TOF/TOF (iTRAQ)	Sera from 50 moderate OA patients, 50 severe OA patients and 50 asymptomatic controls	None	[69]
136 targets detected with 17 proteins identified as more abundant in OA than RA and 16 more abundant in RA than OA.	LC-MALDI-TOF/TOF	SF samples from 20 OA patients and 20 RA patients	FINC, GELS	[70]
13 targets detected with 2 peptides of interest Fib3-1 and Fib3-2 found increased in OA patients compared to control	LC-MS/MS (Ion Trap)	Urine samples from 10 women undergoing knee replacement surgery and 5 healthy women	Fib3-1, Fib3-2	[71]
37 protein peaks in OA patients differed from RA patients. 3 peaks identified as potential biomarkers of OA, only 1 identified.	SELDI-TOF-MS	SF samples from 36 patients with OA and 24 with RA	S100A12	[72]
66 targets detected, differentially expressed in healthy and OA SF	MALDI-TOF/LC-triple	SF samples from 10 control subjects, 10 patients with early-stage OA, and 10 patients with late-stage OA	None	[73]
Apolipoprotein C-I, C-III, and an isoform of transthyretin differed significantly between progressors and non-progressors	SELDI-TOF	Plasma samples from 25 OA patients undergoing radiographic progression; 33 with nonprogression, and 11 healthy donors	None	[74]
252 targets detected. 9 proteins differentially expressed by chondrocyte after IL-1 $\beta$ stimulation	LC-LTQ/ LC-TQ (QconCAT)	Articular cartilage from patient undergoing total knee arthroplasty due to OA	None	[75]
106 proteins quantified for early- OA and 118 for late-OA of which 31 and 38 proteins were differentially expressed	LC-MS/MS ( $O^{16}$ labelling)	SF from 5 patient with early shoulder OA, 4 with late shoulder OA and 5 control individuals	Tenascin, Complement factor D and Aggrecan	[76]

310 targets detected with 55 proteins increased in OA cartilage and 21 decreased in OA cartilage compared with control	LC-MALDI/iTRAQ	Articular cartilage from 16 patients with OA and Articular cartilage from 6 femoral neck fracture for the control group	LECT2, BAALC and PRDX6	[77]
575 targets detected with 92 proteins upregulated and 43 downregulated in RA SF compared to OA	LC-MS/MS (LTQ-Orbitrap / iTRAQ)	SF samples from affected knees of 10 RA and 10 OA patients	CAPG	[78]
677 targets detected among which 545 have not been previously reported	LC-LTQ-FT Orbitrap/ LC-triple quadrupole	SF samples from 10 OA patients	None	[79]
Identification of 12 novel COMP neopeptides from RA, OA or trauma patients	Ion Trap (MS/MS)	SF from patients with acute knee pain, established OA, and established RA	COMP-Ser <sup>77</sup>	[80]
76 targets detected classified in 3 different group profile according to their release from the cartilage	2D-LC-MALDI TOF/TOF (iTRAQ)	Articular cartilage from 4 OA patient undergoing joint replacement and 4 patient with no history of joint disease (N)	Osteoprotegerin, periostin	[81]
~2400 targets detected with 269 showing differential levels between OA and control.	LC- LTQ-Orbitrap	Chondrocytes isolated from articular cartilage of 10 patients OA undergoing knee replacement surgery and 6 normal donors with no history of joint disease	GSTP1, PLS3	[82]
29 targets detected with 22 upregulation proteins and 7 downregulation in the OA group. Haptoglobin protein identified as positively correlated with the severity of OA	2DE/(MALDI-TOF/TOF MS)	SF proteins obtained from 10 knee OA patients and 10 non-OA patients.	Haptoglobin	[83]

**2-DE**: two-dimensional gel electrophoresis; **ANPEP**: alanyl (membrane) aminopeptidase; **BAALC**: brain and acute leukemia cytoplasmic; **CAPG**: macrophage capping protein; **CHST14**: carbohydrate sulfotransferase 14; **COMP-Ser<sup>77</sup>**: cartilage oligomeric matrix protein (neopeptide) serine<sup>77</sup>; **DKK3**: dickkopf WNT signalling pathway inhibitor 3; **ELISA**: enzyme-linked immunosorbent assay; **Fib3-1**: fibulin 3-1, **Fib3-2**: fibulin 3-2; **FINC**: fibronectin; **FT**: Fourier transform ion cyclotron resonance; **GELS**: gelsolin; **GSTP1**: Glutathione S-transferase P1; **LC**: liquid chromatography; **LECT2**: leukocyte cell derived chemotaxin-2; **LTQ**: linear trap quadrupole; **OGN**: osteoglycin, **PLS3**: Plastin-3; **PRDX6**: peroxiredoxin-6; **S100A12**: calcium binding protein A12; **TQ**: triple quadrupole; **WB**: Western Blot.

These recent studies have led to the discovery of many potential new OA biomarkers which undoubtedly will be investigated widely to establish their value as markers of OA in a similar way to the first-generation biomarkers for OA and other joint diseases. From the studies reported in table 2, 10 studies confirmed differential expression of proteins/peptides identified by MS using a different methodology such as western blot, ELISA or immunohistochemistry. The majority of the studies specifically chose to validate their proteins using commercially available antibodies or ELISA kits. Only two studies developed their own antibodies [71, 80].

In order to identify proteins that are differentially expressed between OA and RA, several groups used quantitative proteomic to profile SF obtained from OA and RA patients. A comparative study conducted by Mateo *et al* revealed differential expression of some proteins that could constitute potential disease biomarkers [70]. Particularly, fibronectin and gelosin have been identified as candidate proteins, as their level increased in SF from OA patient and were chosen to be verified for their reported value as biomarkers by western blotting with commercially available antibodies. Han *et al*, also conducted a study comparing SF of OA and RA patients and he too identified a protein of interest upregulated in OA patients compared to RA called S100A12 (calcium binding protein A12) that has been verified by western blotting [72]. Although these specific studies did not include different stages of the diseases and show no comparison with control group patients, recent studies confirmed fibronectin and S100A12 as potential new biomarker for OA. Indeed, in the literature it has been reported by different groups that fibronectin fragment (initially resulting from articular cartilage matrix degradation [84]) is present in high concentration in the cartilage of patients with OA and if added to human cartilage explants culture promoted further degradation of the cartilage [84, 85]. Wang *et al*, reported a study where they correlated the level of S100A12 in serum and SF with the clinical severity of primary knee OA [86]. In addition, another study by a different group demonstrated that S100A12 expression significantly increased in OA cartilages, and consequently promoted the development of OA by up-regulating inflammatory pathways [87]. In 2014, Balakrishnan *et al* reported the largest number of protein identification from SF of OA and RA patients using iTRAQ labelling followed by high-resolution MS analysis. 575 proteins were identified out of which 135 were found to be differentially expressed in both conditions with a few not previously reported to be associated with RA. Only 1 protein CAPG (macrophage capping protein) was confirmed to be up-regulated in RA by WB. All novel proteins identified in these different studies should be explored further as they have been confirmed in different technics and could constitute a new panel of novel biomarkers for OA and RA.

Henrotin and co-workers performed a study using urine samples from women with severe OA undergoing knee replacement and non-OA healthy individuals using LC-MS/MS (Ion Trap) [71]. Thirteen proteins showed significant differential expression between groups. Among these, two peptides named Fibulin3-1 and Fibulin3-2 (Fib), were of particular interest. After generating antibodies to Fib3-1 and Fib3-2, the investigators developed ELISA assays to quantify and validate their peptides of interest using serum from 76 patients with OA and 140 age-matched healthy

subjects. The data from their validation study showed a significant increase of Fib3-1 and Fib3-2 in OA subjects. However, there was no evidence of any disease specificity and the study had a number of limitations including use of patients with severe, end-stage OA, who are not representative of the general OA population.

Another group studied patients with shoulder OA which is less described in the literature in comparison to knee and hip OA and identified a similar pattern of protein expression in these three sites [76]. SF from patients with early and late OA shoulder was analysed and compared to control individual by MS. Wanner *et al* observed 31 and 38 differentially expressed proteins for early and late OA respectively. From these proteins, only 3 (Tenascin, complement factor D and aggrecan) were verified by western blotting and corroborated the MS results.

MS study can be carried out in different biological samples including serum, urine, SF, bone and cartilage. Using the iTRAQ method, Ikeda *et al* identified 76 proteins with differential expression in OA cartilage. From these proteins, they identified three: LECT2 (leukocyte cell derived chemotaxin-2), BAALC (brain and acute leukemia, cytoplasmic), and PRDX6 (peroxiredoxin-6), as potential novel biomarkers for OA [77]. The levels of expression of these proteins were verified by WB using commercially available antibodies on protein extract from cartilage of OA patients and control group. These proteins represent another group of potential new markers of OA but need to be further investigated.

COMP is a marker of cartilage metabolism [88], which is known to be increased in cartilage [89], SF and serum in both OA and RA patients compared to healthy controls [90, 91]. In a recent study using affinity chromatography and MS, Ahrman *et al* have identified twelve different COMP neoepitopes from SF of patients with acute knee pain, established OA, and established RA. One of the neoepitopes Ser<sup>77</sup>, was reported to be elevated in subjects with acute knee pain group compared to the other two groups. Antibodies were raised against this neopeptide and an ELISA assay was developed. The ELISA clearly distinguished between the COMP fragments containing the neoepitope and the total COMP molecules, and therefore may be a suitable for monitoring cartilage degradation [80].



In 2014, Lourido *et al* published a quantitative proteomic analysis using iTRAQ on secretome (molecule released by cultured cells) from healthy human articular cartilage explants, in comparison to explant from different zones of osteoarthritic tissue representing unwounded (early disease) and wounded (advanced disease) zones [81]. A panel of 76 proteins was identified and shown to be differentially released by the OA tissue and the proteins profile released from each cartilage have been classified by cluster analysis into six different groups. The authors reported that osteoprotegerin and periostin proteins were decreased and increased in OA respectively. Osteoprotegerin is an important regulator of bone erosion [92] and known to be involved with periostin in bone remodelling in OA. These proteins were further verified by WB on independent samples from cartilage secretome. Furthermore, the group showed an increase of periostin in SF from OA-patient compared to non-OA patient as well as an increase of the periostin gene expression in OA cartilage which correlated with its abundant release from the OA cartilage explant. However, the authors in this study acknowledge the inconsistencies with previously published data about the levels of osteoprotegerin in OA patients and its correlation with the disease that need to be further investigated. Another MS-based study using proteome of chondrocytes isolated from articular cartilage of patients with OA compared to non-arthritic ones, identified around ~2400 proteins [82]. Amongst these proteins, 269 were differentially synthesised between the two groups and the authors also identified several pathways and proteins to be associated with OA chondrocytes. Only two proteins, GSTP1 and PLS3 (Glutathione S-transferase P1, Plastin-3), were validated by WB and they should be further investigated as potential markers of OA chondrocyte phenotype.

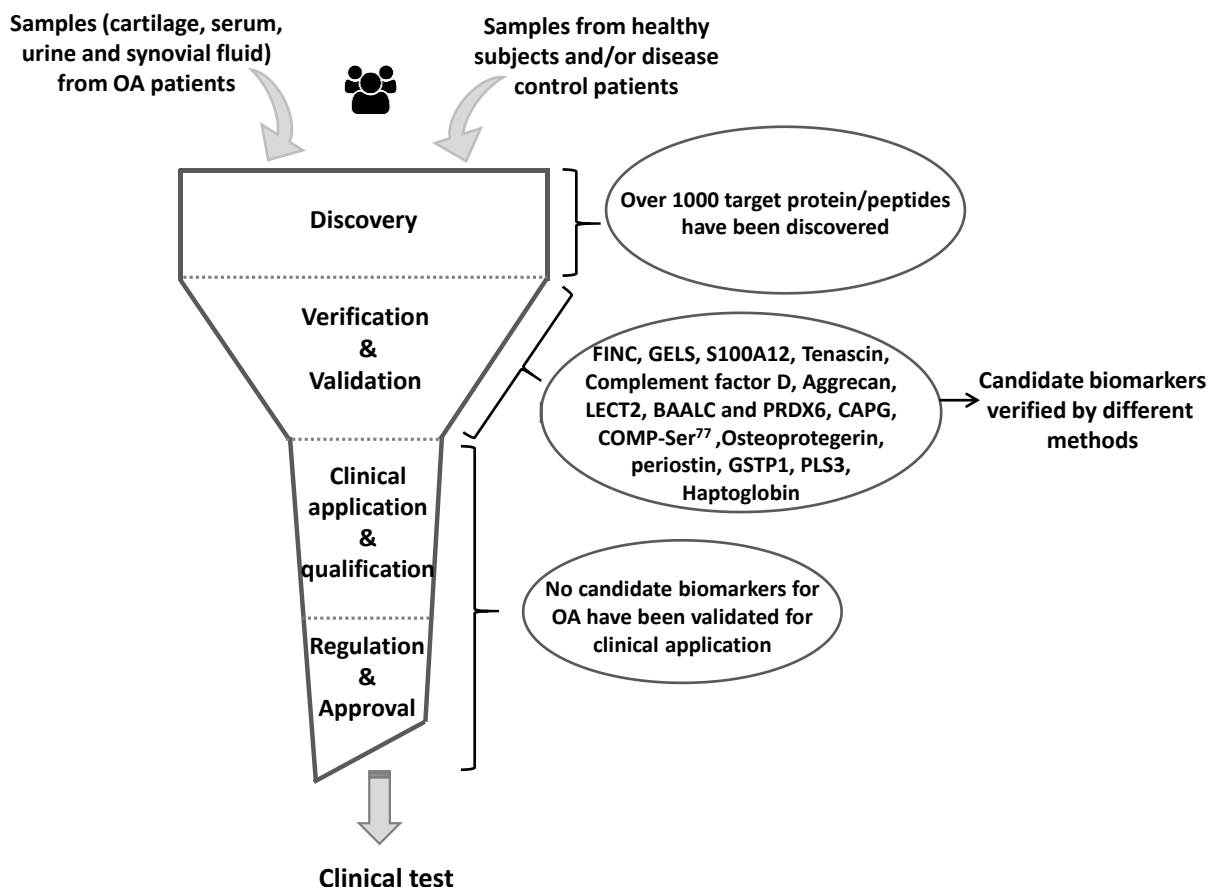
The latest study in table 2 is from Liao *et al* and describes a 2-DE method, followed by protein identification by MS, to look at the proteomic profiles of SF from patients with OA knee compared to non-OA patients [83]. The OA patients were categorised into 6 grades of different OA severity based on the Outerbridge classification. The 2-DE revealed 29 proteins with significant differential expression, with 22 being upregulated and 7 downregulated in the OA group. Only one of the upregulated protein was confirmed to be haptoglobin by MS and by ELISA, and showed that the levels of haptoglobin was positively correlated with the severity of OA. Interestingly haptoglobin has also been identified as a potential OA biomarker in a previous study by Fernandez-Costa *et al* (from Francisco Blanco's group) using a novel sequential depletion strategy coupled with two-dimensional difference in-gel electrophoresis technic [93]. This study showed an upregulation of

haptoglobin in serum of OA patients and confirmed it by WB. All together these data show upregulation in serum and SF of OA-patients making haptoglobin an important protein to investigate as diagnostic marker for OA. Candidate biomarkers such as this one, that have been identified by independent groups with corroborating results should be taken to the next stage of biomarker development and possible clinical application.

We have used MS (SELDI: Surface-enhanced laser desorption / ionisation) to compare serum from normal control patient, OA patients and RA patients, that led to the discovery of 4 new novel biomarkers of OA [67]. Two of the biomarkers, identified as C3f and V65 peptides appeared to be specific for OA patients in comparison to normal control (NC) as well as disease control subjects (RA). C3f and V65 could be detected in non-radiographic stage of OA (Kellgren & Lawrence (K&L) grade 0) and levels increased as the radiographic disease severity increased. The ProteinChip SELDI-TOF used in our proteomic study is limited to high-throughput protein profiling of particularly low molecular weight peptides/proteins (below 20 kDa), and this posed significant technical challenges for raising suitable antibodies to tiny peptides for the development of immunoassays (see later).

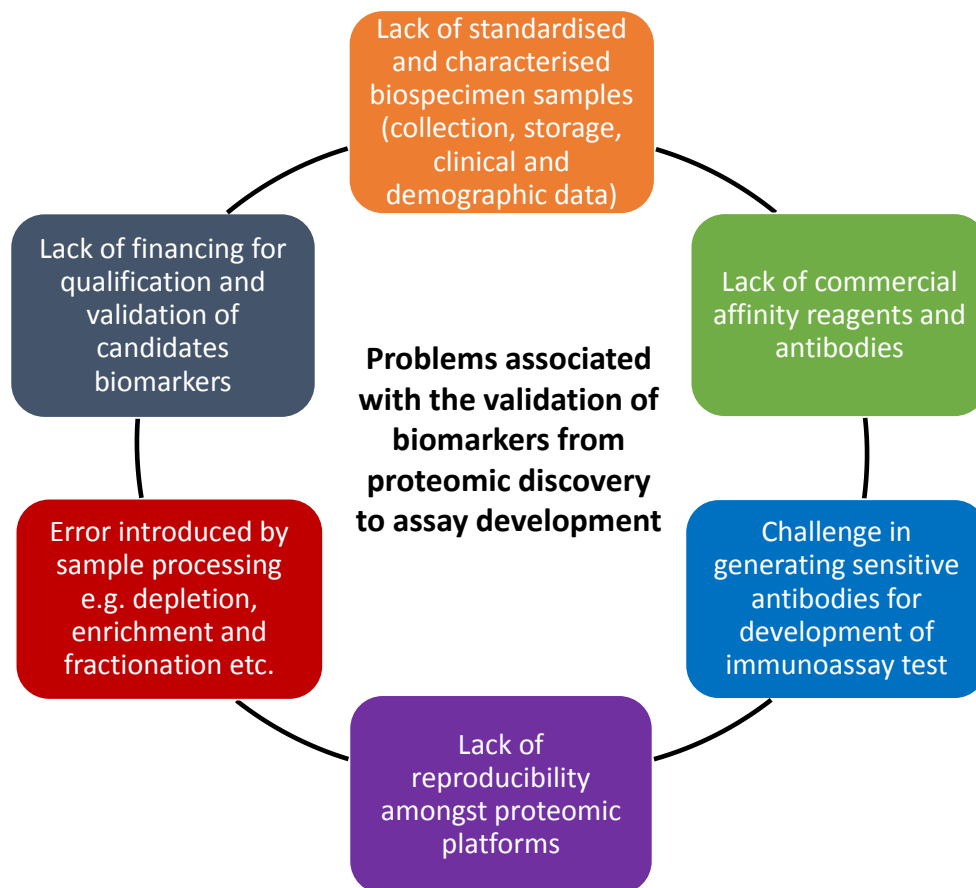
### **Challenges in the validation of biomarkers discovered by proteomic**

The challenge facing the scientific community is the undeniable difference between the discoveries of candidate biomarkers every day and the number of actual biomarkers that reach qualification to be developed as laboratory tests ([figure 1](#)). The question of what is jamming the pipeline between the discovery and the validation stage has been asked many times over the years. [Figure 2](#), summarises the consensual reason that has emerged to explain this difference as well as our one experience in validating biomarkers discovered from MS-based proteomic studies.



**Figure 1:** Current developmental stage of OA biomarkers from MS-based techniques discussed in this article

For successful biomarker qualification and validation, patients and normal control selection, as well as sample collection are crucial. The collection of specimen such as serum or urine samples in most studies is not standardised and many markers levels are affected by demographic variables such as age, gender and BMI. The samples should be collected and stored in a similar way and ideally not defrosted more than once prior to analysis. Samples must be fully characterised with clinical and demographic data and where possible also characterised as progressors or non-progressors (according to x-ray and MRI scores) for OA cohort studies. The use of poorly characterised samples from patients for proteomic analysis is one of the main reason why many of the proteomic OA biomarkers have not progressed beyond the initial discovery phase.



**Figure 2:** Possible reasons for the difficulties in validating biomarkers discovered from MS-based proteomic

The first step in the identification of biomarkers is the selection of potential candidates based on relative association with biological and pathological processes. For OA disease, it might be molecules related to bone remodelling, synovial inflammation or cartilage damage. Commonly the biological samples used for discovery or comparative analysis are serum that usually contains more than  $10^6$ -fold abundance proteins [94] which poses considerable difficulty for the detection of potential proteins of interest that might be present in scarce amount (often nanograms to pictograms). Hence the major requirement in sample processing is to maximise the concentration of the scarce proteins/peptides before mass spectrometric analysis. Sample processing using method for protein separation and purification such as 2-D Clean-Up Kit may also induce loss of potential proteins of interests that are already in very small quantities [95]. Moreover, when potential new markers are detected by MS it's a whole new challenge to acquire the level of sensitivity for the detection of these proteins/peptide in standard assays for the development of diagnostic kits.

MS can detect atom quantities, therefore, it can be a real challenge to extrapolate the detection by mass spectroscopy to simple assays such as ELISA or Western blotting. Secondly, in standard assays, the serum is usually diluted which significantly decreases the amount of the scarce proteins of interest to often below the detection limit of the assays. Therefore, pre-processing of the sample to enrich the target biomarker by depleting the abundant proteins is often required and the sample may also have to be concentrated in order to maximise the chance of detection. Generally, the latter treatments work well with large protein but when dealing with very small protein (less than 2kDa) any pre-processing tends to remove the small proteins. For instance, when developing our ELISA for the detection of C3f and V65, we first depleted the serum of abundant large proteins with a 2kDa cut-off filter then concentrated the samples for analysis. However, depletion of abundant protein to enhance the concentration of the peptides of interest turned out to be far more challenging than we expected. C3f and V65 peptides (<2kDa) did not pass through the filter, they got trapped/lost within the filter membrane. When we spiked with large known concentrations (microgram to nanogram/ml) of the synthetic peptides none of the peptides could be recovered in the filtrate.

Another major problem associated with the development of simple assays for measuring biomarkers derived from MS-based studies is the lack of commercially available antibodies; and the generation of suitable antibodies which can be challenging, time-consuming, and costly and therefore can impair the chance of establishing sensitive immunoassays for diagnostic use. Generally, the proteomic study leads to the identification of potential proteins/peptides candidate biomarkers. Then, in order to be verified, antibodies are produced against these candidates usually by immunising animals with the synthetic peptide, for development of suitable assays. As the exact form of the corresponding peptide/protein in the biological samples is not known, the epitope orientation of the synthetic peptide used for the generation of antibodies could be different from the endogenous one. In addition, the protein/peptide itself could be part of bigger molecules as well as being free. For example, we have identified C3f peptide as a specific biomarker for OA which is a tiny 18 amino acid complement fragment released during the catabolic degradation of C3b after C3 complement activation [67]. Therefore, as C3f is located within the whole C3 and C3b molecules, the development of specific antibodies against C3f posed a great challenge in our recent study. Accordingly, future studies would be advised to consider these issues before embarking on assay development for biomarkers identified from proteomic studies.

Recently, multiple reaction monitoring MS (MRM-MS) also known as selected reaction monitoring (SRM) or targeted MS method, has emerged as an intermediate stage between the discovery and validation of biomarkers [96]. MRM-MS, is used to counter the lack of available affinity reagents and antibodies for those novel protein candidates as well as allowing a better and quicker selection of these proteins. It is used as an accurate and quantitative multiplex filter to verify the specificity and sensitivity of candidates before immunoassay development and validation for potential biomarkers. This “verification” step consists of triaging large amounts of potential novel biomarkers discovered by MS-based proteomics, in biological fluids using targeted quantitative methods [97]. This filtering step ensures that only the most reliable candidate biomarkers progress to the assay development and clinical validation studies. Fernandez-Puente *et al* (From Francisco Blanco’s group) used MRM method for verification and quantitation of a selection of 14 biomarker candidates for OA, identified by MS-based proteomic studies and/or previously reported to be associated with the OA pathology [98]. This panel of proteins was first quantified in five different sample types (human articular chondrocytes, healthy and OA cartilage, SF and serum), and for verification of the results, the authors analysed the 14 potential OA biomarkers in 116 serum samples from OA and healthy controls. The study showed that two of the proteins (haptoglobin and von Willebrand Factor) were significantly increased in OA patients. This study is an example of how MRM can be used for further verification of potential biomarkers. In addition, the authors went on to measure von Willebrand Factor in serum samples from OA patient with K&L grade 2 and 4 and in healthy subjects using Luminex assay for validation of the biomarker as a marker of OA.

As described above, MS can measure large number of abundant proteins and indicate relative changes in abundance for small number of sample. While immunoassays and MRM technologies can measure a small number of lower abundance proteins but none of these techniques can do both. However, new complementary strategies are emerging to overcome this limitation. For instance, a new aptamer-based approach has been developed by using SOMAscan technology. SOMAscan assay is a multiplexed, highly sensitive (dynamic range from femtomolar to micromolar concentrations) platform that simultaneously measures >1000 analytes in biological samples [99]. The SOMAscan assay uses SOMAmer<sup>®</sup> (Slow Off-rate Modified Aptamers) which are single-stranded DNA constructs that bind to native folded proteins with high affinity and have specificity superior to antibody-mediated detection [100]. In addition, fractionation and enrichment of low

abundance proteins are not required as SOMAscan assay is not susceptible to interference from high abundance proteins, making sample preparation much simpler compared to mass spectrometry methods.

#### **4. Conclusions**

Measurements of metabolic biomarkers of cartilage, bone and synovial tissue in biological samples, along with clinical and radiological data from patients, have led us to better understand the pathogenesis and structural changes in OA and RA. However, the currently available biomarkers (first-generation biomarkers) especially for OA have limited value in diagnosis, prognosis or in monitoring effect of treatment. Currently, there is a great deal of interests in MS-based proteomic studies for discovery of biomarker and already thousands of candidates have been identified. However, the MS technologies come with flaws, as they can either measure a large number of abundant proteins, or a small number of lower abundance proteins, but not both. Not to mention the difficulties associated with the validation of biomarkers from proteomic discovery to assay development. Nonetheless, advances in proteomic technology are rapid and new generation technologies may help resolve some of the technical challenges encountered currently. Until then, studies that develop and carry out validation of new candidate biomarkers must be followed through as they represent our best chances to generate new panels of OA biomarker.

The availability of disease specific biomarkers would help us to develop simple, non-invasive tests to be used as point of care diagnostic/prognostic test. Early diagnosis of OA would enable to differentiate between progressive OA (which worsens over time) and non-progressive (stable) to help target treatment to those likely to progress and minimise risks of progression, and therefore delaying or preventing the need for joint replacement surgery. Discovery of a good OA biomarker would involve a long and challenging process from discovery of a potential biomarker to verification and validation process. Hence, the need for better funding is critical for research looking at tools that will overcome the challenges.

#### **Future perspective**

One of the major issues about the biomarkers discussed above is lack of specificity; there is currently no single biomarker that can be considered diagnostic, prognostic or efficacy of

treatment for OA. However, ACAPs may be close to being a diagnostic marker for RA. The second issue is that the collection of samples (cartilage, serum, urine or synovial fluid) in most studies is not standardised and concentrations of many of the biomarkers are affected by demographic variables such as age, gender and BMI. These limitations have to be overcome before we can have a robust set of biomarkers to investigate OA and RA. In addition, we should be mindful of the fact that it may take years to identify a specific biomarker or panel of biomarkers for OA and RA. Moreover, as OA is considered to be a complex and heterogeneous disease it would be unrealistic to expect a single biomarker to identify the presence of OA. As discussed in this review, several candidates OA-biomarker from recent MS-based studies could be potential diagnostic/prognostic marker but these biomarkers need to be properly validated using well characterised cohorts of patients. Moreover, their potential diagnostic/prognostic values need to be evaluated both singly as well as in combination with established biomarkers and clinical and imaging parameters.

### **Executive Summary'**

- Biomarkers specific for RA and OA would enable early diagnosis and better monitoring of patients with these conditions.

### **Diagnosis of OA and RA and possible role of biomarkers**

- Some biomarkers are markers of pathological processes such as cartilage loss and therefore would be useful for monitoring cartilage damage in different conditions including OA and RA.
- The pathogenesis of RA is better defined and understood compared to OA but there is still a need for suitable biomarkers that would help to identify treatment windows, when the patients are likely to respond better to a particular drug.
- None of the first-generation biomarkers for OA is sufficiently specific for diagnosis and monitoring OA.
- The auto-antibody biomarkers (e.g. ACPAs) appear to be better markers of presence of RA.

### **Biomarkers from recent proteomic studies**

- Many potential OA biomarkers have been recently identified using MS-based techniques but none have been validated and verified for use in individual patient.



### **Future perspective**

- The MS-based techniques are very promising tools for discovery of biomarkers but there are a number of technical hurdles that need to be resolved. The availability of MRM-MS may help to overcome at least some of the technical problems associated validation of biomarker discovered by MS studies.

### **Financial & competing interest's disclosure**

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript

### **Disclosure**

This is a narrative review that reflects our personal experience and understanding of the status of the field. Selection of articles was based on our personal judgment of relevance within the scope of this review but did not cover all the studies available.

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