IDENTIFICATION AND VALIDATION OF CANDIDATE SOLUBLE BIOMARKERS FOR PSORIATIC ARTHRITIS USING QUANTITATIVE PROTEOMICS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Laboratory Medicine and Pathobiology
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Abstract

There is a high prevalence of undiagnosed PsA in psoriasis patients; therefore identifying soluble biomarkers for PsA will help in screening psoriasis patients for appropriate referral to a rheumatologist. Potential PsA biomarkers likely originate in sites of inflammation, such as inflamed joints and skin, and subsequently enter systemic circulation. Therefore, we aimed to discover novel biomarkers, to facilitate PsA recognition in psoriasis patients. To achieve this objective, quantitative proteomic analyses of synovial fluid (SF) samples and skin biopsies obtained from PsA patients were performed. SF was obtained from swollen knee joints of 10 PsA patients, and age/sex matched early osteoarthritis (OA) controls. Likewise, skin biopsies were obtained from involved and uninvolved skin of 10 PsA, and 10 age/sex matched psoriasis patients without PsA (PsC). Using strong cation exchange chromatography, followed by tandem mass spectrometry, we characterized the proteomes of pooled SF and pooled skin samples. Extracted ion current intensities were used to calculate protein abundance ratios, and utilized to classify upregulated proteins. Selected reaction monitoring assays were developed to quantify these potential PsA markers in individual patient samples. Verified markers were subsequently measured in serum samples from 100 PsA, 100 PsC patients, and 100 healthy controls, using
commercially available or in-house developed enzyme-linked immunosorbent assays. We quantified a total of 443 and 1922 proteins in SF and skin extracts, respectively, but only 17 proteins represented upregulated proteins in PsA SF, while 47 proteins were specifically elevated in PsA-derived skin. SRM verification confirmed that 12 and 8 proteins were indeed elevated in an independent set of PsA SF and involved PsA skin, respectively. Based on the fold change between PsA and controls, the associated P-values, and the cellular localization, we ranked the proteins, and selected the following putative markers for validation in the serum - M2BP, CD5L, MMP3, CRP, MPO, and ITGB5. Increased levels of CRP, M2BP, MMP3, and ITGB5 were independently associated with PsA, when compared to PsC. ROC analysis of this model showed an AUC of 0.851 [95% CI (0.799, 0.904)]. Thus, serum CRP, M2BP, and ITGB5 are potential biomarkers of PsA in patients with psoriasis.
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Publications arising from this thesis

Articles


Poster Presentations


Contributions

Dr. Daniela Cretu prepared the thesis, and is responsible for the conceptualization and experimental content.

Drs. Eleftherios Diamandis and Vinod Chandran provided supervision, guidance, and expertise, and thus assisted in the successful completion of this work.

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List of Abbreviations

ACE: Angiotensin-converting enzyme
ACN: Acetonitrile
ACPA: Anti-citrullinated protein antibody
ACTB: Beta-actin
ACTH: Adrenocorticotropic hormone
ANA: Anti-nuclear antibody
APCS: Amyloid P component
APOC1: Apolipoprotein C1
APR: Acute phase reactants
AUC: Area under the curve
BSA: Body surface area
C1-2C: Collagen 2-3/4short
C16ORF62: Chromosome 16 open reading frame 62
C2C: Collagen 2-3/4Clongmono
C4BP: C4b-binding protein
CASPAR: Classification criteria for Psoriatic Arthritis
CD5L: CD5-like protein
CHI3L1: Chitinase-3-like protein 1
CI: Confidence interval
CPII: Procollagen 2 peptide
CPN2: Carboxypeptidase N: polypeptide 2
CRP: C-reactive protein
DEFA1: Neutrophil alpha defensin 1
DIP: Distal interphalangeal
DKK1: Dickkopf-1
ELISA: Enzyme-linked immunosorbent assay
ESI: Electrospray ionization
ESR: Erythrocyte sedimentation rate
FA: Formic Acid
FC: Fold change
FDR: False discovery rate
FHL1: Four and a half LIM domain1 1
FT-ICR: Fourier-transform ion-cyclotron resonance
GPS1: G-protein pathway suppressor 1
H2AFX: Histone 2A type I A
H4: Histone 4
HLA: Human leukocyte antigen
HPLC: High-performance liquid chromatography
IL: Interleukin
ITGA5: Integrin alpha 5
ITGB5: Integrin-beta 5
LC-MS/MS: Liquid chromatography and tandem mass spectrometry
LFQ: Label free quantification
LZIC: Leucine Zipper And CTNNBIP1 Domain-Containing Protein
M2BP: Galectin-3-binding protein
MALDI: Matrix-assisted laser desorption ionization
M-CSF: Macrophage colony stimulating factor
MHC: Major histocompatibility complex
MMP3: Matrix metalloproteinase 3 Stromelysin 1
MPO: Myeloperoxidase
MS/MS: Tandem mass spectrometry
MS: Mass spectrometry
MTX: Methotrexate
NSAID: Non-steroidal anti-inflammatory drugs
OA: Osteoarthritis
OPG: Osteoprotegerin
ORM1: Orosomucoid 1
PAFAH1B2: Platelet-activating factor acetylhydrolase 1b: catalytic subunit 2
PASI: Psoriasis Area and Severity index
PBS: Phosphate buffered saline
PFN1: Profilin 1
PIP: Proximal interphalangeal
POSTN: Periostin
PPP2R4: Protein phosphatase 2A activator: regulatory subunit 4
PsA L: Lesional Psoriatic Arthritis
PsA N: Non-lesional Psoriatic Arthritis
PsA: Psoriatic arthritis
PsC L: Lesional Psoriasis
PsC N: Non-lesional Psoriasis
PsC: Psoriasis
RA: Rheumatoid arthritis
RANKL: Receptor activator for nuclear factor κB ligand
RF: Rheumatoid factor
ROC: Receiver operating characteristic
RP: Reverse phase
S100A9: Protein S100A9
SAA1: Serum amyloid A1
SCX: Strong cation exchange
SD: Standard deviation
SF: Synovial fluid
SNCA: Alpha synuclein
SRM: Selected reaction monitoring
SRP14: Signal recognition particle 14kDa
SRPX: Sushi-repeat containing protein
TFA: Trifluoroacetic acid
TGF: Transforming growth factor
TMB: 3:3′:5:5′-Tetramethylbenzidine
TNF: Tumor necrosis factor
TNFSF14: TNF superfamily member 14
TOF: Time-of-flight
TUBB: Beta-tubulin
XIC: Extracted ion current
Chapter 1

Sections of this chapter have been published in Critical Reviews in Clinical Laboratory Sciences:

1 Introduction

1.1 Psoriasis

1.1.1 Statistics and epidemiology

Psoriasis is an immune-mediated inflammatory skin disease, characterised by scaly, red and well-demarcated skin plaques, resulting from keratinocyte hyperproliferation and altered differentiation, the presence of an inflammatory cell infiltrate and neovascularisation (1). The prevalence of psoriasis varies with race and geographical location, with an estimated prevalence of 3% in North America (2, 3). The lowest prevalence has been reported in Asia, aboriginal Australians, Native American Indians, and West Africans (0-0.3%) (2, 3). Based on age of onset, two types of psoriasis have been described: Type I psoriasis, which has a peak onset between age 20-30, and Type II psoriasis, which develops after the age of 40 (3, 4). Type I psoriasis is the most common, as onset before the age of 40 occurs in up to 75% of patients, but it also takes a more severe course when compared to Type II psoriasis, which tends to be more mild (4, 5).

Severe psoriasis is associated with an increased risk of mortality, whereby male and female patients appear to die 3.5, and 4.4 years earlier, respectively, when compared to age matched controls (6). Psoriasis is also associated with increased mental health disease and suicidal ideation in patients (5, 7). In a recent study, the adjusted hazard ratio of depression was higher in patients with severe psoriasis compared to patients with mild psoriasis (8).
1.1.2  Aetiology

Psoriasis results from the interplay between genetic and environmental factors. Many population and family studies have shown higher incidence of psoriasis in relatives. Evidence supporting genetic predisposition was demonstrated by twin studies, which showed higher concordance rates in monozygotic twins (65-72%), when compared to dizygotic twins (15-30%) (9). The absence of 100% concordance in monozygotic twins, and the lack of a clear inheritance pattern in families, indicates the presence of possible environmental triggers in those who are genetically susceptible (9). As a result, much research has been conducted to understand the genetic and environmental basis of psoriasis. Several loci and genes (PSORS1-10, HLA-C*0602, IL12B, IL12R, TRAF3IP2) have been identified through linkage analysis, and by gene-association studies (2, 10, 11). Additionally, environmental triggers identified include streptococcal infection (12), physical trauma (13), medication (14), smoking (15), and alcohol (16).

1.1.3  Clinical features

Psoriasis is identified by the presence of characteristic plaques (or lesions), which are well-circumscribed red, raised, scaly skin lesions. The redness results from increased growth and dilatation of superficial blood vessels (17, 18). The epidermis of a psoriatic lesion is thicker, and the epidermal rete is elongated due to abnormal proliferation of keratinocytes, which is known as psoriasiform hyperplasia (17, 18). The characteristic scales seen in psoriatic lesions are formed by the rapid maturation and hyperproliferation of epidermal keratinocytes. Lesions are also rich in activated T-lymphocytes which release pro-inflammatory cytokines, leading to the characteristic inflammation seen in the disease (17, 18).
Psoriasis vulgaris occurs in 85-90% of cases, and represents the most common type of psoriasis. It usually affects young adults with plaques involving the scalp, extensor aspect of the elbows, knees, and back (5). Guttate psoriasis is characterized by the acute onset of many small psoriatic lesions, (approximately 1-10mm in diameter). It typically occurs 1-2 weeks following a streptococcal infection, and mainly affects children and young adults (9, 12). Inverse psoriasis is distinct, since it does not exhibit the characteristic plaques, and it affects flexures, typically armpits, groin, and under the breast. These lesions do not present with scales, and appear as red, shiny, and well demarcated plaques. Erythrodermic psoriasis results in a scaling, itching, inflammatory process involving most of the body surface. This may occur as a result of chronic plaques which progress and become confluent, or it may result from unstable psoriasis due to infection, drugs, and stress (5). Erythroderma occurs in 3% of psoriasis patients, and its complications are highly life-threatening, since patients may develop severe infections, pneumonia, and cardiac failure (5, 19). Pustular psoriasis is characterized by white, non-infectious pustules, surrounded by red skin. There are two types of pustular psoriasis: von Zumbusch, or generalized pustular psoriasis, and palmo-plantar pustulosis. The von Zumbusch type is rare, and unstable, where flare-ups occur in repeated waves lasting days or weeks (5, 19).

Finally, psoriatic nail disease is seen in 40-45% of patients with cutaneous psoriasis (5). The most common finding is pitting of the nails, resulting from psoriatic involvement of the nail bed. The nail may also detach from the nail bed distally or laterally (known as onycholysis) (5). Psoriatic nails tend to be deformed and thickened and also exhibit yellow-brown discoloration (5). Psoriatic nail disease is particularly relevant to psoriatic arthritis, as will be discussed later (20).
1.1.4 Diagnosis, measurement, and treatment

Psoriasis is most often diagnosed by history and physical examination, as no diagnostic laboratory tests are available (21). The Psoriasis Area and Severity Index (PASI) and the Body Surface Area (BSA) tools are used in assessing the severity of the disease. PASI combines scoring the severity of the lesions, and the area affected into a single score, whereby 0 indicates no disease, and 72 indicates maximal disease (22, 23). A PASI score greater than 10 indicates severe psoriasis. BSA represents an estimate of the percent body surface that is affected by psoriasis. A BSA score greater than 10% indicates severe psoriasis (22, 23). Treatment varies based on the severity of the disease. Topical corticosteroids, tar, retinoids, and Vitamin D derivatives are generally used for mild psoriasis. Severe disease is treated with phototherapy, and systemic agents such as, methotrexate, cyclosporine, apremilast, or biologic agents, such as, anti-TNF-α, anti-IL12/23 or anti-IL-17 agents (24-26).
1.2 Psoriatic Arthritis

1.2.1 Statistics and epidemiology

Psoriatic arthritis (PsA) is defined as a rheumatoid factor-negative inflammatory arthritis, associated with psoriasis (27). Moll and Wright demonstrated a 19-fold increase in psoriasis prevalence, amongst first-degree relatives with PsA, when compared to the general population (28, 29). The most recent estimate of the prevalence of PsA in North America is 0.25% (6), and the incidence of PsA in the general population ranges from 3.23.1 per 100,000 (30). PsA occurs in around 30% of patients with psoriasis (2, 31-33). Most commonly, psoriasis precedes PsA, but arthritis may also precede the psoriasis (32). Approximately 70% of the patients develop psoriasis before arthritis, and psoriasis and PsA develop simultaneously in an additional 15%, while 15% develop the arthritis before the detection of psoriasis (34). Males and females are equally affected by both, psoriasis and PsA (33). The onset of PsA occurs between the ages of 30-55 years, and most studies report no relationship between the type or severity of skin disease, and the joint manifestations as most patients with PsA have mild or moderate skin disease (35), although patients with more severe psoriasis are at a higher risk of developing (6).

1.2.2 Aetiology and Pathogenesis

There are several factors such as genetic, immunological, and environmental that have been proposed to be of importance for the aetiology and progression of PsA, and this section will discuss the similarity and differences between psoriasis and PsA.

As in psoriasis, genetic factors contributing to the susceptibility for PsA have been analyzed by linkage and association studies (9, 29, 35, 36). A number of familial studies have suggested
first-degree relatives to be at risk of developing PsA (9, 29, 34). A parental gender effect has been demonstrated in both psoriasis and PsA, whereby more patients have an affected father, rather than an affected mother. Several studies of the major histocompatibility complex region on chromosome 6p, have found that HLA-C*06 are more prevalent among patients with psoriasis and PsA, when compared to healthy controls, especially in patients with Type I psoriasis (36-39). When comparing patients with PsA with those with psoriasis alone, HLA-B*08, HLA-C*12, HLA-B*27, and HLA-B*38 alleles were found to indicate an increase in the odds of developing PsA, while HLA-C*06 was found to decrease these odds (40-42). Additionally, HLA-B*27 has been associated with axial involvement, while HLA-B*38 and B*39 have been associated with peripheral disease (40-45); it has been demonstrated that HLA-B*27 represents a strong genetic marker for PsA among psoriasis patients (41, 42). HLA-DQ3 has been suggested as a marker for disease progression (43), and an increased frequency of DR4 has been associated with disease severity, development of polyarticular symmetrical arthritis, and with joint erosions (43, 46, 47).

Non-HLA genes that map close to the MHC region of chromosome 6p, such as MHC class I chain-related gene A (MICA), is in linkage disequilibrium with HLA-B alleles and is reported to have an increased frequency in psoriasis and PsA patients (48-51). Cytokine-related genes, such as IL-23R, IL-12p40, IL23p19, IL-21, IL-4, IL-5, and IL-13, have all been associated with PsA (36, 52).

In addition to genetic factors, immunological mediators have also been described in the pathogenesis of both psoriasis and PsA. In psoriatic skin lesions, increased levels of CD4 T-lymphocytes are found in the dermis (53), while in PsA CD8 T-lymphocyte population is
significantly increased in patients’ synovium (54). Synovial fluid (SF)-derived CD8 T-cells are mature (expressing CD45RO), activated (expressing HLA-DR), and express low levels of CD25 (which represents the α chain of the IL-2 receptor) (55). Additionally, the cytokine profile in PsA is characterized by the presence of Th1 cytokines such as interleukin (IL)-1β, IL-2, interferon-λ, TNF-α, and IL-10 (56). More recently, a number of studies have demonstrated that IL-17, IL-22 and IL-23 are increased in psoriatic skin lesions and the synovium of PsA patients, and have a role in the pathophysiology of these diseases by inducing hyperproliferation of keratinocytes and promotion of synovitis (57-59). These studies were further supported by the fact that inhibition of these cytokines demonstrated clinical benefits of both PsA and psoriasis alone (24, 60). Macrophages, and increased levels of metalloproteases have also been documented in PsA (56, 61, 62). These are just a few factors which are known to play a role in initiating and maintaining the inflammatory milieu observed in PsA and psoriasis patients; a comprehensive description of the inflammatory mediators of PsA is given by Wittmann et al. (26).

Environmental factors playing a role in the aetiology and progression of PsA have been difficult to separate from the immunological factors (63). Initially, due to increased levels of antibodies against bacterial cell wall peptidoglycan found in PsA patients, it was believed that bacterial infections had a role in PsA pathogenesis (12, 54, 55, 64). However, the Th1 cytokine pattern suggests that synovial inflammation is not only driven by an immune response to a bacterial antigen (35, 56). A number of groups have shown that physical injury more often triggers PsA than other arthritis diseases; this is known as the Koebner phenomenon (65-67). The underlying reason for this is not known, but the idea that the release of neuropeptides which can stimulate the synovial membrane and lead to hypervascularization, has been proposed (68). Lifting heavy
loads and smoking have also been associated with the occurrence of arthritis among psoriasis patients, but no association has been found between PsA and alcohol consumption, vaccination, stress, or female hormonal exposures in the most recent study (65).

1.2.3 Clinical Features

PsA has a heterogeneous pattern and patients can present with various symptoms such as mild mono-oligo arthritis or very severe, erosive, and destructive polyarthritis (32, 69). The frequently involved joints are, distal interphalangeal (DIP) and proximal interphalangeal (PIP) joints, the wrists, the metatarsophalangeal joints, the joints of the lower extremities, the sacroiliac joints, and the spinal column (32, 69).

Enthesitis is a characteristic feature of PsA, with inflammation at tendon or ligament attachment sites. Interestingly, MRI detection of enthesitis in clinically uninvolved joints led to the suggestion that enthesitis may be the primary lesion in PsA, which is also supported by the observation that enthesial inflammation may extend as far as the synovial cavity (70, 71).

Dactylitis, or inflammation involving a complete digit, is also characteristic of PsA, occurring in 48% of the patients. Dactylitis has been associated with worse radiographic appearance (20, 72-74). These extra-articular symptoms differ from patients with rheumatoid arthritis (RA), but do occur among patients with other spondyloarthritides. Other manifestations in patients with PsA include uveitis, distal extremity swelling, and discoloration of the skin over affected joints, and inflammatory bowel disease (75-78). Radiological changes in PsA include: erosion of terminal phalangeal tufts, whittling of bone ends, cupping of proximal ends of bones, severe destruction of isolated small joints, sacroiliitis, or syndesmophytes (69, 75). During recent years it has
become apparent that PsA can be a very destructive disease, and approximately 20% of patients develop severe, deforming arthritis (79).

1.2.4 Relationship between the skin and joint

Since in most patients psoriasis precedes PsA, the skin disease most often serves as a marker for subjects at risk of developing PsA. As described previously, immune cells and the mediators they secrete influence disease initiation and progression in psoriasis and PsA. In psoriasis, trauma may lead to skin lesions, which is reflected by the lesion pattern seen in psoriasis (35); areas repeatedly exposed to trauma or pressure, such as knees and elbows, often develop plaque psoriasis (13, 67). Interestingly, in PsA, DIP joints are often involved with adjacent psoriatic nail disease. Thus, it has been proposed that inflammation of the nail/skin, initiates inflammation in the closest joint leading to arthritis or enthesitis, in patients that are susceptible. Alternatively, it has also been suggested that with the extensor tendon enthesitis linking the joint to the nail bed, the involvement of the nail bed may be due to extending inflammation from the joint (35). It is still unclear, which of the two proposals are correct.

1.2.5 Diagnosis of PsA

The diagnosis of PsA is considered when inflammatory arthritis, spondylitis or enthesitis occurs in patients with psoriasis. The criteria defined by Moll and Wright were used as ‘diagnostic criteria’ over the last three decades (68). There were no universally agreed upon classification criteria until the ClASsification of Psoriatic Arthritis (CASPAR) criteria were developed (27). The CASPAR criteria have high sensitivity and specificity for PsA (91.4% and 98.7%),
respectively), but require a rheumatologist to determine whether a patient has inflammatory musculoskeletal disease (80, 81) (Table 1.1).

**Table 1.1 The CASPAR criteria for classification of PsA**

<table>
<thead>
<tr>
<th>Inflammatory articular disease (joint, spine, enthesal) with more than 3 points from the following categories:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Current (2 points) or past (1 point) presence of skin psoriasis, or a family history of psoriasis (1 point)</td>
</tr>
<tr>
<td>2. Psoriatic nail lesions (1 point)</td>
</tr>
<tr>
<td>3. Dactylitis (1 point)</td>
</tr>
<tr>
<td>4. Negative rheumatoid factor (1 point)</td>
</tr>
<tr>
<td>5. Radiographic evidence of juxta-articular new bone formation (1 point)</td>
</tr>
</tbody>
</table>

**1.2.6 Treatment**

Non-steroidal anti-inflammatory drugs (NSAIDs) are the basic treatment for the pain, and stiffness experienced in mild arthritis. In NSAIDs non-respondents, traditional ‘disease modifying anti-rheumatic drugs (DMARDs)’ such as, sulfasalazine, methotrexate, leflunomide, and cyclosporine, are used and often effective against arthritis, either as single treatments or in combination (22, 82-84). Newer agents that include the phosphodiesterase inhibitor apremilast and biological agents, such as anti-TNF-α and IL 12/23 agents, have improved signs and symptoms of PsA and psoriasis (24, 26, 82, 85, 86).
1.2.7 Identifying PsA Early

As mentioned previously, the presence of psoriasis indicates a high risk for the existence or future development of PsA since 85% to 90% of patients with PsA have psoriasis at the onset of PsA. Early diagnosis and management of PsA leads to better long-term outcomes (87-89). In a recent survey conducted in Canada they found that although 18% of patients were diagnosed with PsA the number who reported joint pain or stiffness was 51%, suggesting that a number of patients may have had early or undiagnosed PsA (90). Additionally, other studies conducted in dermatology clinics have also shown a high prevalence of undiagnosed PsA in patients with psoriasis (72). Therefore dermatologists have the potential to play an important role in preventing joint destruction in psoriasis patients, by screening for signs of PsA, initiating treatment, and referring patients to a rheumatologist if needed (91). Unfortunately, for dermatologists, identifying inflammatory musculoskeletal disease early in patients with psoriasis is a very difficult task. Although there are clinical features that may help predict PsA, such as nail, scalp and intergluteal/perianal psoriasis, and psoriasis severity, these symptoms are non-specific (80, 92). Additionally, acute phase reactants may be normal, and there are no validated biomarkers. Therefore much of the recent research in PsA, has focused on identifying, serum-based biomarkers. These laboratory tests could be used to screen psoriasis patients, in order to identify those that may have or develop PsA; patients that screen positive can then be referred to a rheumatologist for appropriate investigation and treatment (10, 11, 93).
1.3 Biomarker discovery in rheumatology

1.3.1 Definition of biomarkers

The National Institutes of Health Biomarker Definition Working Group, defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or a pharmacologic response to a therapeutic intervention” (94). As such, these biomarkers can be used in the clinic to diagnose, predict disease progression, monitor activity of the disease, assess therapeutic response, or guide molecular targeted therapy (94). Biomarkers for joint diseases may be clinical, histological or imaging features, as well as genomic, proteomic, and transcriptomic markers (10, 11, 76, 93, 95). Table 1.2 outlines examples of commonly used serum-based markers for various joint diseases (96-105). Apart from classical acute phase reactants, the limitations of which will be discussed later, such a biomarker does not currently exist for PsA, and the current need lies in identifying a prognostic serum-based marker (or markers), which can be used to screen psoriasis patients to determine those that are at risk of having or developing PsA (10, 11, 93, 95).
Table 1.2 Examples of soluble biomarkers used in the diagnosis and treatment of joint diseases

<table>
<thead>
<tr>
<th>Marker</th>
<th>Molecular Class</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>Metabolite</td>
<td>Drug toxicity (98)</td>
</tr>
<tr>
<td>CRP</td>
<td>Protein</td>
<td>Identify acute inflammation (99)</td>
</tr>
<tr>
<td>ANA</td>
<td>Autoantibody</td>
<td>Diagnostic for various joint diseases (100)</td>
</tr>
<tr>
<td>RF</td>
<td>Autoantibody</td>
<td>Diagnostic for RA (101, 102)</td>
</tr>
<tr>
<td>ACPA</td>
<td>Autoantibody</td>
<td>Diagnostic and prognostic for RA (103, 104)</td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>Autoantibody</td>
<td>Diagnostic for SLE (105)</td>
</tr>
</tbody>
</table>

1.3.2 Ideal PsA biomarkers

Ideal PsA biomarkers should be produced by cells within involved tissues, such as synovial fibroblasts in the inflamed joint, or keratinocytes in the psoriatic epidermis, and enter systemic circulation where they can be detected. Thus, the ideal PsA biomarker should not already be present in high concentration in the blood of healthy individuals, and in patients with psoriasis without arthritis, since the biomarker contributions from PsA will be difficult to measure. Finally, ideal markers should demonstrate high sensitivity and high specificity.

1.3.3 Advantages of protein biomarkers

The foremost advantage of studying proteins is that the actual functional molecules of a cell are being investigated, elucidating a reliable picture of what is occurring in the tissue/joint. Proteins are more diverse than nucleic acids, since alternative splicing and post-translational modifications may result in multiple proteins from one gene (112, 113). Further, many
physiologic changes are mediated post-transcriptionally, and therefore will not be reflected at the nucleic acid level (112, 113). Proteins are also more dynamic, and more reflective of cellular physiology (113). For example, a double stranded DNA break results in a cascade of protein phosphorylation. Finally, and most importantly in the context of biomarkers, proteins are more stable and easily measureable, compared to mRNA which can be rapidly degraded (114). Therefore, protein biomarkers carry several advantages over genomic and transcriptomic biomarkers.

1.3.4 Serum biomarkers in PsA

Serum protein biomarkers represent the most cost-effective, non-invasive, and objective way to detect, stage, prognosticate, and monitor disease activity and response to treatment. A number of putative PsA biomarkers have already been identified, but to date, none have been validated in a clinical setting. The types of serum protein PsA biomarkers may be divided into acute phase reactants, markers of cartilage repair and destruction, inflammatory markers, markers of bone destruction and new bone formation, and markers of extracellular matrix destruction. As it will become apparent, PsA is a highly heterogeneous disease, and it is unlikely that a single marker will prove sufficient in serving as an ideal biomarker (10, 11, 93).

1.3.4.1 Acute phase reactants

Acute phase reactants (APR) represent serum proteins that increase or decrease in response to inflammation. APRs such as C-reactive protein (CRP) and Erythrocyte Sedimentation Rate (ESR) are only elevated in 50% of PsA, even in the presence of active disease. A highly-sensitive CRP (hsCRP) assay has been developed, whereby CRP is measureable in levels
<5mg/l. This assay has shown a lot of promise, since it was demonstrated, in a pilot study, that hsCRP levels in PsA are higher than those in psoriasis (10, 11, 93, 115, 116).

1.3.4.2 Markers of cartilage repair and destruction

Cartilage destruction and repair is characteristic of inflammatory arthritis, and the products of synthesis and destruction are released in the serum where they can be measured (117). Cartilage oligomeric matrix protein (COMP), a glycoprotein expressed in cartilage, tendons, synovial membrane, and scarified skin, is also found to be elevated in PsA, and correlated strongly with inflammatory parameters and number of inflamed joints (115). Unfortunately, COMP was also elevated in psoriasis patients, and a difference between levels of COMP in PsA and psoriasis was not observed (115, 118). Additionally, the articular cartilage is composed of Type II collagen network complexed with aggrecan (115, 119). It is known that cleavage of type II collagen in articular cartilage by collagenases generates neoepitopes Col2-3/4C$_{\text{long mono}}$ (C2C) and Col2-3/4C$_{\text{short}}$ (C1-2C) (115). As Type II collagen is degraded, chondrocytes respond by upregulating production of procollagen (115). The procollagen peptide (CPII) is cleaved when procollagen is secreted; therefore the rate of type II collagen synthesis is directly proportional to the amount of CPII in the cartilage (115). While neither of the peptides are informative on their own, the ratio of CPII to C2C (CPII:C2C) represents the balance between type II collagen synthesis and degradation. In a small-scale study, increased levels of CPII:C2C were independently associated with PsA, when compared to psoriasis alone [OR(95%CI)=4.76(1.35,16.77)] (115). Additional validation studies are still underway.
1.3.4.3 Cytokines and chemokines

Inflammation is the hallmark of PsA, and several inflammatory markers have also been proposed. Chandran et al. investigated the ability of IL-12p40 to distinguish between PsA and psoriasis (115). Although the levels of IL-12p40 were higher in PsA, compared to controls, the elevation was not significant (115). Cumulative evidence strongly supports the involvement of IL-23/IL-17 axis in the pathogenesis of PsA, and a number of compounds that target components of these pathways have been recently used in PsA clinical trials (24, 82, 120). IL-23 acts synergistically with IL-6 and TGF-β to promote rapid Th17 cell development and IL-17 release (121, 122), which in turn, plays a central role in sustaining chronic inflammation (121). Serum IL-6 levels are elevated in PsA patients compared to psoriasis, and correlate well with joint counts, ESR, CRP, and IL-2Rα (11, 93, 116). Additionally, in a recent study, IL-17 secretion was elevated in both PsA and psoriasis when compared to healthy controls, with no significant difference between PsA and psoriasis, while IL-22 expression was 2-fold higher in PsA when compared to psoriasis without arthritis (57). Additional studies are needed to show reproducibility of IL-22 expression.

1.3.4.4 Markers of bone destruction and new bone formation

Bone resorption, mediated by osteoclasts, and new bone formation, mediated by osteoblasts, has been increasingly investigated in the context of PsA. Osteoclast activity is modulated by macrophage colony stimulating factor (M-CSF), the receptor activator for nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG). In turn, TNF superfamily member 14 (TNFSF14) promotes RANKL-dependent osteoclastogenesis. In the same study by Chandran et al., OPG, TNFSF14, and RANKL were measured in patients’ serum, but only elevated OPG was found to
have discriminatory ability between PsA and psoriasis [OR(95%CI)=1.01(1.00,1.02)] (115).

OPG is a cytokine that inhibits osteoclast differentiation and promotes new bone formation. It is thus believed to be a marker of periostitis and new bone formation, the combination of which represents a characteristic that differentiates PsA from other inflammatory arthritides (27). Additionally, Dickkopf-1 (DKK-1), a regulatory molecule of the Wnt signalling pathway, inhibits osteoblast function and is also a key regulator of joint remodelling (123). DKK-1 levels are increased in PsA compared to psoriasis without arthritis (124). Its elevation has also been demonstrated in patients with active rheumatoid arthritis (123), casting doubt on the role and significance of circulating DKK1 in PsA; further studies are thus warranted.

1.3.4.5 Markers of extracellular matrix destruction

Matrix-metalloproteinase 3 (MMP3) is involved in the breakdown of extracellular matrix and tissue remodelling in normal, as well as pathologic conditions. In PsA, as well as other rheumatic conditions characterized by synovitis, MMP3 is involved in the destruction of cartilage and bone. In a pilot study, serum MMP3 levels were increased in patients with PsA, and could be used to discriminate between PsA and psoriasis without arthritis [OR(95%CI)=1.28(1.02,1.60)] (115). These results are preliminary, and require validation in a larger study.

1.3.5 Proteomics in biomarker discovery

Genomic and transcriptomic studies have been robust and led to the identification of a number of susceptibility genes and expression profiles in PsA (10, 11, 93, 106). Proteomic studies to identify serum protein biomarkers, represent a more recent approach to PsA biomarker discovery, and has the potential to complement genomics-based approaches by bridging the gap
between what is encoded in the genome and what is occurring at the tissue/joint level (107). It is well known that genomic and proteomic data sets have different sources of bias and variance, so combining them may lead to a more precise view of the differential protein abundance (108, 109). The key benefit of the integration of proteomic and transcriptomic data in the field of biomarker discovery is its potential for improving the selection of candidates to validate. If both transcriptomic and proteomic platforms agree on a strong differential expression between the groups of patients being compared, the attractiveness of a candidate is strengthened (110, 111).
1.4 Proteomic methods used in discovering putative biomarkers in rheumatology

Mass-spectrometry (MS) is a technique that measures the mass-to-charge ratio of ions, to identify and quantify molecules in simple and complex mixtures. MS has become an invaluable tool across many fields and applications, including proteomics. The initial development of high-throughput qualitative, and more recently quantitative proteomic methods, have expanded our knowledge of protein structure, function, modification, and profiles in disease states.

1.4.1 Basic components of a mass-spectrometer

MS is performed on a mass-spectrometer, which consists of an ionization source, a mass analyzer, and an ion detector. The specifics of these components, the type of data generated (qualitative, quantitative, post-translational modifications, etc.), and the physical properties of the samples that can be analyzed, vary across different instruments (125).

Following standard sample preparation procedures which typically includes protein denaturation, reduction, alkylation, and peptide separation, samples are loaded into the mass-spectrometer in liquid form, and the next step is to convert the sample into gas-phase ions using the ionization source. Ionization sources can vary largely, but are typically classified as either electrospay ionization (ESI), or matrix-assisted laser desorption ionization (MALDI) (125). In ESI, ions are produced at atmospheric pressure by running the sample through a narrow capillary tube in an electrostatic field. The resulting electric potential difference generates a fine mist of charged droplets. The solvent is evaporated, typically using Nitrogen gas, and along with Coulombic forces, nanometer-sized droplets are produced (125). Alternatively, MALDI ions
result from combining sample molecules with small organic molecules which are capable of absorbing light when irradiated with a laser beam. The matrix absorbs light at the wavelength of the laser, which leads to desorption and ionization of both, the matrix and sample. MALDI is well-suited for analyzing samples containing analytes greater than 200kDa, while ESI allows analysis of smaller analytes (less than 1KDa). Therefore, the choice of ionization depends largely on the nature of the sample being analyzed (125).

The charge received from ionization allows the mass spectrometer to accelerate the ions through the remainder of the instrument. The gas-phase ions enter the mass analyzer, where they encounter electrical or magnetic fields, which deflect the paths of individual ions based on their mass to charge ratio (m/z). Mass analyzers can also vary, and include quadrupole time-of-flight (Q-TOF), triple quadrupole, orbitrap, quadrupole ion traps, TOF, and TOF/TOF, and Fourier-transform ion-cyclotron resonance (FT-ICR) analyzers. Ion-trap mass analyzers utilize magnetic and radio frequency to hold ions, while quadrupole ion-trap analyzers use an oscillating electric field for ion storage and mass analysis (125). Using the electrode, the orbitrap mass analyzer traps ions in an electrostatic field, causing the ions to move in a spiral pattern. The quadrupole analyzer consists of four parallel metal rods, where each opposing rod pair is electrically connected. Ions are separated based on their trajectory in the oscillating electric fields that are applied to the rods (125). In a triple quadrupole (QQQ), the first quadrupole (Q), and the last quadrupole act as mass analyzers, while the second quadrupole is used as a collision cell where peptides are fragmented. The performance of a mass analyzer depends on the maximum allowable mass that can be analyzed (mass range), the smallest amount of analyte that is detected with high confidence ( detection sensitivity), and its ability to separate two mass ions (resolution) (125).
Ions successfully passing through the mass analyzers are then detected. The detectors are, most often, electron multipliers or microchannel plates, which emit electron cascades when each ion hits the detector plate. This results in the amplification of each ion, and improves sensitivity (125). The detector measures the electric current in proportion with the number of ions striking it, and generates a mass spectrum, which is analyzed using various pattern-matching algorithms.

### 1.4.2 Sample fractionation methods

The dynamic range in protein concentration in many biological fluids and tissues extends from mg/mL for abundant structural and carrier proteins such as albumin and keratin, to pg/mL for signaling molecules such as TNF-α. This is similar to plasma, where the concentration range is approximately 12 orders of magnitude. The proteomic analytical methods currently available have a concentration range within 2-5 orders of magnitude, leading to low detection of less abundant proteins (126-129). A common way to overcome this problem and reduce protein complexity is by implementing pre-fractionation methods and/or depleting the sample of the most abundant proteins using depleting columns. Proteins bind directly to the column or indirectly through secondary binding to immunoglobulins or albumin (or other high-abundant proteins). Although all commercially available columns have been designed for serum and plasma, they are also valuable for other biological fluids such as synovial fluid and ascetic fluid, because most abundant proteins in these fluids correspond to those in serum/plasma (130). It is important to note that, high-abundance proteins can physically mask less abundant proteins with similar isoelectric points and molecular weights. Depletion columns may also bind proteins in a non-specific manner (131, 132). Depletion should only be used when whole sample integrity is not essential. According to a study by Chen et al., treatment of synovial fluid prior to
fractionation will decrease reproducibility and increase protein loss; therefore, it should also be avoided (133). Instead, low molecular weight components of samples, which may represent putative biomarkers, can be enriched by size exclusion liquid chromatography (LC), strong cation/anion exchange (SCX/SAX) LC, or reverse phase (RP) LC. In size exclusion chromatography, molecules are separated based on their size or molecular weight. This method is generally good for separation of large molecules from small ones, but it can result in approximately 50% total protein loss. SCX and SAX lead to the separation of analytes based on their charge. These represent the most frequently used separation methods, as they have a high resolving power, capacity, and simplicity. Finally, in RP-LC molecules are separated by hydrophobicity, whereby proteins are eluted using a gradient of hydrophobic solvent. This method is commonly used as a last separation step following sample preparation and prior to MS analysis.

1.4.3 Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) offers additional information about ions being analyzed. In this instance, ions of interest are selected based on their m/z from the first round of MS (MS1), and these are then fragmented, usually via collisions with inert gas atoms, as is the case in collision-induced dissociation. The resulting fragments are then separated based on their individual m/z in an additional round of MS (MS2) (125, 129). This process ultimately allows the identification and characterization of proteins.
1.4.4 Quantitative Mass-Spectrometry Methods

Quantification of protein levels to achieve accurate differential protein profiling between biological samples has been a major challenge in proteomics. These methods require chemical labeling of the samples prior to MS analysis, or can be performed label-free, termed label-free quantification (LFQ). More recently, targeted quantitative mass-spectrometry methods have been developed, in the form of selected reaction monitoring (SRM) assays (134-136).

1.4.4.1 Label-free quantification

The label-free protocol is semi-quantitative, and is based on the peak intensity of the peptides in the MS scan, or on the number of observed spectra per peptide across different samples (125). In general, the advantage of label-free approaches over chemical labeling lies in the low cost and the high number of samples that can be easily included in the experiment (136). Potential disadvantages of label-free techniques include the lower reproducibility of results, which compromises detection of smaller quantitative changes between samples (136). In terms of data analysis, spectral counting is the simplest label-free method but is also the least reliable because quantification accuracy drops when the number of spectra representing a protein becomes very low, usually less than two (136). As a result, classifying smaller changes among the identified low abundance proteins, which typically have low numbers of observed spectra, will be difficult with this method. Alternatively, intensity-based quantification is, in theory, more capable of obtaining accurate values for lower abundance proteins (137). Computer algorithms such as MaxQuant are used to analyze the data, and require the identification of only one peptide in at least one of the samples being analyzed to extract the peak intensity information and to quantify the peptide in all the analyzed samples (138, 139).
1.4.4.2 Chemical labeling

As an alternative to label-free approaches, chemical labeling of peptides/proteins prior to fractionation and MS analysis has also been used. There are a number of available labeling strategies: tandem mass tags (TMT) (140), isobaric tags for relative quantification (iTRAQ) (141), and isotope-coded affinity tags (ICAT) (142) are the most popular commercial alternatives. In the case of iTRAQ (4-plex and 8-plex) and TMT (6-plex), free amines generated from trypsin digestion and present in all the peptides are labeled and, therefore, theoretically no information is lost (136). Reporter ions relating to the various samples being analyzed are released from the peptides during MS/MS fragmentation and are thus used to represent the sample from which specific proteins originate; no quantitative information is obtained from the MS scan (140, 141). These methods are not without challenges, as utilizing these protocols in complex samples may result in the partial suppression of the quantitative reporter ion signal (143). Low-abundance proteins are more vulnerable to this effect, and unfortunately these are also the proteins that represent potential biomarkers. ICAT only labels cysteine residues therefore proteins that do not have peptides containing cysteine, will not be quantified. In addition, the quantitative information from each protein with this method is sparse, and ICAT only appears as duplex labels (143). Generally, chemical labeling protocols are rather lengthy and involve many steps, resulting in compromised reproducibility.

1.4.4.3 Selected reaction monitoring

Recently, there has been a paradigm shift toward the use of targeted MS-based methods. SRM assays exploit the capabilities of triple quadrupole or Q-Trap instruments (135). For reliable quantification of a protein of interest, proteotypic peptides or peptides unique to a particular
protein of interest, are first selected. The corresponding predefined precursor masses of these peptides are selected in the first quadrupole and fragmented in the second quadrupole, with predefined fragmentation masses selected in the third quadrupole. This unique pair of precursor and fragmentation mass is termed a transition. The SRM method can be applied simultaneously to multiple proteins (MRM), a protocol that is further reviewed by Lange et al. (135). Stable Isotope Dilution-SRM (SID-SRM) is based on the selection of three to five peptides resulting from tryptic digestion from each protein to be quantified (144). Synthetic peptides containing heavy lysine and arginine residues (which have incorporated 13C/15N atoms) are then added to all samples. These peptides serve as internal standards providing relative quantitative ratios for each proteotypic peptide corresponding to each protein of interest between all samples (144). The nature of this approach allows for very high-molecular selectivity, and if interference is present it can also be detected (144).

**1.4.5 Biomarker development pipeline**

Three different phases exist in the discovery of novel biomarkers: discovery, verification, and validation (145). In the discovery phase, a small number of well-characterized, high-quality samples are compared using fractionation and quantitative proteomics methods to generate an extensive list of protein components. Subsequent phases in the biomarker development pipeline replace the unbiased experimental design with target-driven quantitative strategies relying mainly on target-driven analytical methods. During the verification phase, preliminary assays are employed, such as enzyme-linked immune sorbent assays (ELISA) and selected reaction monitoring assays, to measure the levels of selected proteins in relevant biological samples. To advance the development, all biomarker candidates also require validation, which is undertaken
on only a subset of verified candidates and is performed in, ideally, thousands of samples (145). This stage requires the development of robust immunoassays to measure the proteins accurately in serum samples. Potential biomarkers showing good sensitivity and specificity are considered for further clinical evaluation (145).

1.4.6 Sources to mine for PsA biomarkers

In the context of PsA, blood obtained by venipuncture is the most accessible, minimally invasive, and the most practical human specimen that can be monitored over long periods of time. Blood plasma contains proteins shed from all organs and tissues. However, performing mass spectrometric analysis of plasma or serum analysis presents with several challenges. These include high complexity in the number of proteins and protein isoforms, a concentration range between high-abundance and lows-abundance proteins of 12 orders of magnitude, and changes in protein concentration, structure, and function resulting from physiological and pathological processes. Therefore, the discovery of biomarkers from serum by mass-spectrometry analysis has proven to be a challenging task (126-128).

Fortunately in the case of PsA, the affected joint offers access to synovial fluid. SF is a potential source of valuable markers. Synovial fluid is obtained from affected patients by arthrocentesis (joint aspiration). Synovial fluid is secreted by the synovial membrane, and is in direct contact with both the synovial membrane, and the articular cartilage (146). Consequently, SF contains specific additions made from proximal joint tissue, including the synovial membrane and cartilage, therefore changes in the cellular metabolism and structure of these tissues as they occur in a disease state may be reflected by changes in SF composition (146). This particular characteristic can be exploited when searching for biomarkers of any joint disease. In a study by
Mateos *et al.*, SF was utilized to mine for RA biomarkers using MALDI-TOF, which resulted in the identification of 136 differentially expressed possible RA biomarkers (147). The synovial membrane also represents an excellent reservoir of possible PsA biomarkers. Obviously the synovial membrane has an advantage over synovial fluid since it is the site of inflammation in PsA, but these samples, as well as appropriate controls are hard to obtain. Another important source to investigate for potential biomarkers is the skin, as it represents another PsA target tissue. The premise is that certain proteins originating/elevated from this tissue during disease, could subsequently enter the bloodstream. The inflammatory process, as it occurs in PsA, is characterized by vascular changes, such as vasodilation, increased permeability and increased blood flow (148). These processes may facilitate shedding or secretion of skin proteins into the blood stream. In a 2011 study, tandem mass-spectrometric analysis of psoriatic skin tissue showed differential expression of 146 proteins in lesional (affected) psoriatic skin, when compared to nonlesional (unaffected and healthy skin) (149). Although these markers were never validated, this study provides evidence that proteomic signatures differ between lesional and nonlesional skin.
1.5 Rationale and Aims of the Present Study

1.5.1 Rationale

The presence of cutaneous psoriasis is a high risk for developing PsA, but several studies that have been conducted in dermatology clinics have shown a high prevalence of undiagnosed PsA in psoriasis patients. This is due to the fact that identifying inflammatory musculoskeletal disease early in patients is difficult since the symptoms are non-specific, and acute-phase reactants are often normal. While there are clinical features that can be utilized to predict PsA, such as nail, scalp, intergluteal/perianal psoriasis, and psoriasis severity, these are common among patients with psoriasis. Identifying biomarkers that can recognize PsA in psoriasis patients may help in early diagnosis and subsequent prevention of disability and improvement in quality of life.

There is no doubt that genomic and transcriptomic methods are powerful, as they resulted in the identification of a number of significant susceptibility genes and expression profiles in PsA. But, proteins are more diverse and carry more information than nucleic acids. Post-translational modification and alternative splicing may lead to numerous protein variants from the same gene. Additionally, information provided by nucleic acids is limited, as they are unable to predict downstream events, such as what protein, and in what quantities will be expressed in a particular tissue or fluid in a pathologic state. Candidate PsA protein biomarkers have been selected for validation based on their assumed importance in disease pathogenesis, and the availability of assays, but to date, the need for discovery of soluble PsA biomarkers in psoriasis patients has remained unmet. Classical acute phase reactants, such as ESR and CRP are the only laboratory tests currently used in clinic, but these are non-specific markers of inflammation that have poor
sensitivity and specificity. Therefore, analyzing proteins can provide additional information, by relating specific proteins to a disease such as PsA.

MS-based proteomic approaches are well-suited for the discovery of protein mediators of disease. While early studies relied on qualitative analysis, more recently, semi-quantitative and quantitative comparisons of protein abundance are the preferred methods for identifying differentially expressed proteins in biological samples.

Blood obtained by venipuncture is the most practical human specimen that can be used to monitor disease status over long periods of time. However, high throughput mass-spectrometry analysis of serum or plasma has proven to be a difficult task. Due to its close proximity to the diseased joint, synovial fluid represents an ideal source to mine for potential PsA biomarkers. Additionally, since skin is the other important target tissue in PsA, proteins elevated in the skin may also present candidate PsA biomarkers.

1.5.2 Hypothesis

Quantitative mass spectrometry-based proteomic analysis of synovial fluid and skin tissue from PsA patients and appropriate controls, will generate a comprehensive list of proteins specific to PsA, facilitating the identification of candidate PsA screening biomarkers.
1.5.3 Specific aims

1) To perform high-throughput label-free quantitative proteomics to identify upregulated PsA-specific proteins in synovial fluid.

   i) Develop sample preparation method best suited for label-free quantification analysis of synovial fluid.

   ii) Utilize bioinformatics tools to filter and select the most informative proteins.

   iii) Develop selected reaction monitoring assays and verify candidate markers in individual synovial fluid patient samples.

2) To perform high-throughput label-free quantitative proteomics to identify upregulated PsA-specific proteins in skin.

   i) Develop sample preparation method best suited for label-free quantification analysis of skin.

   ii) Utilize bioinformatics tools to filter and select the most informative proteins.

   iii) Develop selected reaction monitoring assays and verify candidate markers in individual skin patient samples.

3) To validate verified markers in PsA serum samples using enzyme-linked immunosorbent assays.
The data presented in this chapter has been published in Clinical Proteomics:

2 Quantitative proteomic analysis of psoriatic arthritis synovial fluid

2.1 Introduction

Psoriatic arthritis is an inflammatory arthritis distinguished by bone resorption and periarticular new bone formation, and bears its name from its association with the cutaneous disease, psoriasis (150). PsA occurs in approximately 30% of psoriasis patients and in about 85% of cases psoriasis precedes or occurs simultaneously with PsA (2, 6, 32). PsA has a predicted prevalence of 0.16 to 0.25% in the general population, and is a complex, potentially disabling musculoskeletal disorder often arising early in age. Patients with PsA are also at increased risk of co-morbidities, such as obesity, metabolic syndrome, diabetes, and cardiovascular disease (31, 32). The aetiology of psoriasis and PsA remains unclear, but studies indicate that interaction between multiple genetic components and environmental factors are important in the disease pathogenesis (2, 9, 65, 106, 151). It is proposed that environmental factors such as infections by Streptococci or articular trauma (2, 41, 42, 65, 67, 152, 153) may trigger immunological alterations in genetically predisposed individuals that play important roles in the appearance of both skin and articular disease. From the immunological point of view, changes are observed in both innate and adaptive immunity. Undoubtedly, the identification of key PsA mediators will not only provide valuable information towards a deeper understanding of the molecular basis of the disease, but it might also uncover important PsA biomarkers potentially useful for clinical follow-up and response to treatment.

Mass spectrometry-based proteomic approaches are well-suited for the discovery of protein mediators of disease. Early studies relied on qualitative (identity-based) analysis, and
performance was depended mainly on the sensitivity of the available MS platforms and sample processing prior to MS-analysis (154, 155). More recently, semi-quantitative and quantitative comparisons of protein relative abundance are the preferred methods for identifying differentially expressed proteins (156). In many cases, chemical or metabolic labeling of samples prior to analysis has been utilized for quantitation, but it has been associated with technical challenges (136). Label-free quantification (LFQ) methods have also been recently optimized, in which quantification is based on the differential peak intensity [extracted ion current (XIC)] of the peptides in each MS scan (125, 157).

LFQ quantitative proteomics presents a robust means for obtaining proteome profiles of virtually any biological material (125, 157). Human plasma represents a diverse proteome and is an excellent source for protein mediators of disease, but proteins secreted by adjacent tissues are diluted in blood, and are often undetectable by current MS methods (97). To circumvent this issue, attention has been focused on proximal fluids, such as ascites (158), and seminal fluid (159) to search for tissue-associated markers. For instance, in the case of PsA, as mentioned previously, synovial fluid (SF) represents an interesting source of PsA-related proteins secreted by the synovium, ligament, meniscus, articular cartilage, and joint capsule. Moreover, it is well known, that there is an exchange of proteins between SF and the systemic circulation through the synovial lymphatics and vasculature (129). In support of this, we have demonstrated that proteins elevated in the SF of PsA patients, are likewise upregulated at the serum level (160). We have therefore decided to focus on SF for the discovery of putative PsA biomarkers.

In the present study, we performed label-free MS quantitation of SF proteins from PsA and early osteoarthritis (OA). Using a highly sensitive and specific MS-based approach, we confirmed the
elevation of specific elevated proteins in an independent set of samples from patients with PsA. These observations may shed new light on the pathogenesis of PsA, offer insights into disease progression, and might reveal potential PsA biomarkers.
2.2 Methods

2.2.1 SF Proteomic analysis

2.2.1.1 Human subjects and clinical samples

The study received institutional review board approval from the University Health Network, and informed consent was obtained from all patients.

For the discovery phase, SF was obtained from 10 cases with PsA (6 males, 4 females; age range 30-76 years), and 10 age- and sex-matched controls (early OA) (Set I). PsA patients had psoriasis and satisfied the CASPARE classification criteria (27). Inclusion criteria included symptom duration ranging from 1 to 10 years (to capture both relatively early and established disease), and at least one inflamed and accessible large joint. The inflammatory nature of the SF, and the absence of other causes of inflammation, such as infection and/or crystal disease, was confirmed by laboratory investigations.

SF from joints with early OA was obtained during an arthroscopic procedure. Early OA was defined as only a partial thickness cartilage defect in any compartment of the knee, and further defined by a grade I or II lesion by the Outerbridge classification (161).

For the verification (quantification) phase, an independent set of SF samples (Set II) was acquired from 10 PsA patients (7 males, 3 females; age range 21-66 years), and 10 age- and sex-matched early OA controls. Inclusion and exclusion criteria were the same as described above.
2.2.1.2 Pre-analytical sample processing

Synovial fluid samples were stored at -80°C until use. Samples were centrifuged upon thawing at 1800g for 10 minutes, to remove any debris, and the total protein was measured in each sample using the Coomassie (Bradford) total protein assay (Pierce Biotechnology, IL). Equal protein amounts of each SF sample were combined, to obtain two (1mg total) pools (PsA vs. early OA), which were analyzed in triplicates. Proteins in each pool were denatured using heat (95°C for 10 minutes), reduced with 5mM dithiothreitol at 60°C for 45 minutes, and alkylated with 15mM iodoacetamide, in the dark at room temperature for 45 minutes. Sequencing grade trypsin (Promega, WI) was added in a 1:50 (trypsin: protein) ratio, and allowed to digest for 18 hours at 37°C. The samples were subsequently acidified (pH 2) using 1uL of formic acid, to inhibit trypsin activity. The resulting peptides were then subjected to high-performance liquid chromatography (HPLC) using strong cation exchange (SCX) columns to reduce peptide complexity.

2.2.1.3 HPLC-SCX

Digested samples were diluted 1:2 in mobile phase A SCX buffer (0.26M formic acid (FA), 10% acetonitrile (ACN); pH 2-3) and loaded directly onto a 500µL loop connected to a PolySULFOETHYL A column (2.1 mm × 200 mm; 5 µ; 200 °A; The Nest Group Inc., MA), containing a silica-based hydrophilic, anionic polymer (poly-2-sulfothethyl aspartamide). An Agilent 1100 HPLC (Agilent Technologies, Germany) system was used for fractionation. A 60-minute gradient was employed with a linear gradient starting at 30 minutes and consisting of mobile phase A and mobile phase B (0.26M FA, 10% ACN, 1M ammonium formate; pH-4.5)
for elution of peptides (flow rate 200µL/min). The fractionation was monitored at a wavelength of 280 nm, and performed in triplicate. Fractions were collected every two minutes from 20 to 55 minutes, and those with a low peak absorbance were pooled, resulting in a total of 10/15 fractions per sample (10 fractions for early OA replicates, and 15 fractions for PsA replicates). This amounted to a total of 75 SCX fractions, which were then subjected to liquid chromatographic and tandem mass spectrometric analysis (LC-MS/MS). SCX column and system performance was ensured by running a quality control peptide mixture consisting of 1µg/µL Alpha Bag Cell peptide, 1µg/µL Fibrinogen fragment, 5µg/µL Human ACTH, and 5µg/µL ACE Inhibitor (American Protein Company, CA) after every sample.

2.2.1.4 LC-MS/MS

The SCX fractions were purified through C-18 OMIX Pipette Tips (Agilent Technologies, Germany), to remove impurities and salts, and eluted in 5µL of 65% MS buffer B (90% ACN, 0.1% FA, 10% water, 0.02% Trifluoroacetic Acid (TFA)) and 35% MS buffer A (5% ACN, 0.1% FA, 95% water, 0.02% TFA). The samples were diluted to 85µL in MS buffer A, and injected into a nano-LC system (Proxeon Biosystems, FL) connected online to an LTQ-Orbitrap (Thermo Fisher Scientific, USA). A 90 minute linear gradient reversed phase chromatography using MS buffer A and MS buffer B, was performed at a flow rate of 400nL/min to resolve peptides on a C-18 column (75µM x 5 cm; Proxeon Biosystems, FL). The MS parameters were: 300 Da minimum mass, 4000 Da maximum mass, automatic precursor charge selection, 10 minimum peaks per MS/MS scan; and 1 minimum scan per group. XCalibur software v.2.0.7 (Thermo Fisher Scientific, USA) was used for data acquisition.
2.2.1.5 Protein identification and quantification

Raw files corresponding to early OA, and PsA data sets were uploaded into MaxQuant v. 1.2.2.2 (www.maxquant.org) (139) and searched with Andromeda (built into MaxQuant) (138) against the nonredundant IPI.Human v.3.71 (86, 309 sequences; released March 2010) database, which contains both forward and reverse protein sequences. Search parameters included a fixed carbamidomethylation of cysteines, and variable modifications of methionine oxidation and N-terminal acetylation. Data was initially searched against a “human first search” database with a parent tolerance of 20 ppm and a fragment tolerance of 0.5 Da in order to calculate and adjust the correct parent tolerance to 5 ppm for the search against the IPI.Human fasta file. During the search, the IPI.Human fasta database was randomized and false detection rate was set to 1% at the peptide and protein levels. Data was analyzed using “Label-free quantification” checked, and the “Match between runs” interval was set to 2 min. Proteins were identified with a minimum of one unique peptide. “LFQ Intensity” columns corresponding to the extracted ion current (XIC) value of each protein in replicate early OA and PsA groups were averaged and used to calculate PsA/early OA ratios (fold change; FC).

2.2.1.6 Bioinformatics analysis

To minimize false positives, we excluded from further analysis any protein with an individual false detection rate >0.05. Proteins displaying an XIC value lower than 100,000 were regarded as absent (noise). We also excluded proteins present in only one of the three technical replicates. Averages of the technical replicate XIC values were calculated for each PsA and early OA group, and ratios of PsA/early OA were used to identify deregulated proteins. Upregulated
proteins were denoted with a PsA/early OA ratio (FC) greater than 2, while downregulated proteins had a PsA/early OA ratio (FC) less than 0.8. Housekeeping proteins were represented with a PsA/early OA ratio (FC) of approximately 1.0. To determine the possible origin of differentially expressed proteins at the tissue level, and identify the most probable mediators of PsA, gene names were checked against gene [BioGPS (http://biogps.org/#goto=welcome) (162), and protein [Human Protein Atlas (http://www.proteinatlas.org/) (163) databases, to identify proteins with strong expression in PsA-associated tissues and cell types (skin, bone, immune cells). The Plasma Proteome Database (http://www.plasmaproteomedatabase.org/) (126) was employed to identify proteins present in high-abundance in the serum, which could represent potential contaminants. Selected reaction monitoring (SRM) assays were developed for the top upregulated proteins in the PsA group and housekeeping proteins, and relative protein quantification was performed in individual SF samples to confirm their elevation in PsA SF.

2.2.1.7 Network analysis

The list of dysregulated proteins identified by LC-MS/MS was analyzed by pathway analysis using the network-building tool, Ingenuity Pathways Analysis (IPA; Ingenuity Systems, www.ingenuity.com) (164).

2.2.2 Verification of identified proteins using SRM assays

SRM methods were developed for verification of protein ratios in SF, following our in-house protocols (165).
2.2.2.1 SRM assay development

PeptideAtlas (http://www.peptideatlas.org/) was utilized to select the most commonly observed 3-4 tryptic peptides for the proteins of interest. Their presence was confirmed using our LC-MS/MS identification data. The uniqueness of peptides was verified using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). To identify peptide fragments (transitions) to monitor, in silico peptide fragmentation was performed using Pinpoint software (Thermo Fisher Scientific, USA) and 5-6 transitions were selected for each peptide. For method optimization, digested pooled samples of SF used in our LC-MS/MS analysis, were loaded onto a C-18 column (Proxeon Biosystems, FL) coupled to a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific, USA), and approximately 350 transitions were monitored in 6 subsequent runs. Three transitions of the most intense peptides (per candidate protein) were used for subsequent quantification assays.

2.2.2.2 SF sample preparation

Fifty µg of total protein of each SF sample, were denatured by heat, reduced, alkylated, and trypsin-digested as described previously. In the independent SF sample set (Set II), heavy-labelled versions of the peptides of interest (JPT Peptide Technologies, Germany) ranging from 1 to 1000 fmol/µl of sample were also added, as internal standards. Heavy peptides had identical sequences to the endogenous peptides, except the C-terminal lysine or arginine was labeled with $^{13}$C and $^{15}$N. The resulting peptides were purified through C-18 OMIX Pipette Tips (Agilent Technologies, Germany), and eluted in 3µL of 65% MS buffer B (90% ACN, 0.1% FA, 10% water, 0.02% TFA) and 35% MS buffer A (5% ACN, 0.1% FA, 95% water, 0.02% TFA).
Samples were diluted to 40µL of MS buffer A, randomized, and loaded onto a C-18 column coupled to a triple quadrupole mass spectrometer.

2.2.2.3 SRM assays

SRM assays were developed on a triple-quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific, USA) using a nanoelectrospray ionization source (nano-ESI, Proxeon Biosystems, FL), as previously described (165). Briefly, a 60-minute, three-step gradient was used to load peptides onto the column via an EASY-nLC pump (Proxeon Biosystems, FL), and peptides were analyzed by a multiplexed SRM method using the following parameters: predicted CE values, 0.002 m/z scan width, 0.01 s scan time, 0.3 Q1, 0.7 Q3, 1.5 mTorr Q2 pressure and tuned tube lens values. Quantification, in SF set I, was executed after normalization against a set of 4 peptides corresponding to 2 housekeeping proteins (LRG1, CFI), to offset technical variations. Each sample was analyzed in duplicate, using a 60-minute method, whereby 117 transitions were monitored. Quantification in SF set II was executed following normalization against the added heavy-labelled peptides, as described earlier. Each sample was analyzed in duplicate, using a 60-minute method, whereby 134 transitions were monitored. Reproducibility of the SRM signal was confirmed by running a quality control solution of 0.1 fmol/µL bovine serum albumin, every 10 runs.

2.2.2.4 SRM protein quantification

Raw files recorded for each sample were analyzed using Pinpoint software (Thermo Fisher Scientific, USA) (166), and peptide XICs were extracted. Pinpoint was used for identification and visualization of transitions, as well as manual verification of co-elution of heavy and
endogenous peptides. In the first SF set, in order to control for technical variation between the samples, XIC corresponding to each endogenous peptide replicate were divided by the XIC corresponding to the average of the two housekeeping proteins. This value was then averaged amongst the two replicate runs, to obtain “XIC Average normalized to housekeeping proteins”.

In SF set II, in order to control for technical variation between the samples and obtain a more robust quantitative value for our proteins of interest, the XIC value corresponding to each endogenous peptide, was divided by the XIC value corresponding to each spiked-in heavy peptide, in order to obtain a L:H (light:heavy) ratio. Since we added a known amount of each heavy peptide to our samples prior to analysis, we used the L:H ratio to calculate the relative concentration of each endogenous peptide corresponding to our proteins of interest.

2.2.2.5 Statistical analysis

Results were analyzed using nonparametric statistics with the Mann-Whitney U test. P-values (P) less than 0.05 were considered statistically significant.

Figure 2.1 outlines the overall experimental workflow.
Figure 2.1 Summary of the biomarker discovery and verification experimental workflow in SF
2.3 Results

2.3.1 Delineating the PsA SF proteome

Our LC-MS/MS analysis of SF identified and quantified 443 proteins, which were present in at least two of the three technical replicates representative of each PsA and OA pool. Of these, 137 proteins were differentially regulated (2.0<XIC ratio<0.8) between the PsA and early OA SF, as shown in Table 2.1. A total of 44 proteins were upregulated with a PsA/OA ratio greater than 2, while 93 proteins were downregulated, with a ratio less than 0.8. The IPA software was used to identify dysregulated functional pathways associated with both the upregulated and downregulated proteins in the PsA SF proteome. The top five molecular and cellular functions associated with upregulated proteins were, cell-to-cell signalling and interaction, cell movement, antigen presentation, cell cycle, and cell morphology, some of which have been shown to be attributes of PsA. The top canonical pathways associated with these proteins were acute-phase response signalling, granulocyte adhesion and diapedesis, and production of nitric oxide and reactive oxygen species in macrophages (Table 2.2). While some of the functions were similar, the downregulated proteins were less associated with inflammatory processes. Since our ultimate goal is to identify candidate biomarkers for PsA, we decided to focus on proteins that were overexpressed in PsA SF.
Table 2.1 Differentially expressed proteins between early OA and PsA groups identified by LC-MS/MS

<table>
<thead>
<tr>
<th>Upregulated Proteins</th>
<th>PsA:OA FC</th>
<th>Downregulated Proteins</th>
<th>PsA:OA FC</th>
</tr>
</thead>
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<td>Anti-RhD monoclonal T125 gamma1 heavy chain</td>
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According to gene ontology functional annotation, the majority of these proteins were extracellular, plasma membrane-associated, or proteins with unknown localization (Figure 2.2), consistent with the fact that SF is a proximal fluid, and most of the proteins are likely shed or secreted by chondrocytes, synoviocytes, and inflammatory cells which come in direct contact with this biological fluid. Since inflammation-driven cell death naturally occurs during PsA, this could lead to the release of cytosolic proteins into the SF, therefore the cytosolic proteins we identified also hold biological importance. We focused on proteins displaying strong expression in skin, bone, and immune regulatory cells (Table 2.3), but excluded immunoglobulins from further analysis. This reduced our list to 20 proteins, from which, high abundance serum proteins, as identified using the Plasma Proteome Database, were excluded (SAA1, APCS, APOC1), as we assumed that these were most likely serum contaminants from the joint aspiration or arthroscopic procedure. This filtering yielded a final set of 17 proteins (Table 2.4), which we deemed likely to be associated with PsA. The validity of our discovery approach was further enhanced by the discovery of previously investigated PsA- associated proteins (CRP, MMP3, S100A9) (115, 167, 168, 169).
### Table 2.2 Summary of Ingenuity Pathway Analysis (IPA)-generated functional pathways and diseases associated with elevated proteins identified from PsA SF

<table>
<thead>
<tr>
<th>Diseases and Disorders</th>
<th>Number of Components Identified</th>
<th>Proteins</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connective Tissue Disorders</td>
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<td>ACTA1, DEFA1, IGHM, PLS2, MMP1, MMP3, MPO, S100A9</td>
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<tr>
<td>Inflammatory Disease</td>
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<td>Molecular and Cellular Functions</td>
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<td>Cellular Movement</td>
<td>8</td>
<td>CTSG, DEFA1, SAA1, PLS2, MMP1, MMP3, S100A9, PFN1</td>
<td>5.69E-05</td>
</tr>
<tr>
<td>Antigen Presentation</td>
<td>1</td>
<td>PLS2</td>
<td>2.11E-03</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>1</td>
<td>PFN1</td>
<td>2.11E-03</td>
</tr>
<tr>
<td>Cell Morphology</td>
<td>2</td>
<td>PLS2, PFN1</td>
<td>2.11E-03</td>
</tr>
<tr>
<td>Physiological System Development and Function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematological System Development and Function</td>
<td>10</td>
<td>CTSG, FGA, PLS2, S100A9, MPO, ORM1, DEFA1, SAA1, APCS, IGHM</td>
<td>2.86E-06</td>
</tr>
<tr>
<td>Immune Cell Trafficking</td>
<td>10</td>
<td>CTSG, FGA, PLS2, S100A9, MPO, ORM1, DEFA1, SAA1, MMP1, APCS</td>
<td>2.86E-06</td>
</tr>
<tr>
<td>Tissue Development</td>
<td>8</td>
<td>CTSG, FGA, PLS2, S100A9, MPO, ORM1, SAA1, ACTA1</td>
<td>2.86E-06</td>
</tr>
<tr>
<td>Cell-mediated Immune Response</td>
<td>3</td>
<td>CTSG, DEFA1, PLS2</td>
<td>1.51E-03</td>
</tr>
<tr>
<td>Organismal Survival</td>
<td>2</td>
<td>ACTB, PLS2</td>
<td>3.33E-03</td>
</tr>
<tr>
<td>Top Canonical Pathways</td>
<td>5</td>
<td>Genes Involved</td>
<td>p-value</td>
</tr>
<tr>
<td>-----------------------------------------------------------------</td>
<td>---</td>
<td>--------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LXR/RXR Activation</td>
<td>5</td>
<td>APOB, APOC1, FGA, ORM1, SAA1</td>
<td>2.70E-06</td>
</tr>
<tr>
<td>Acute Phase Response Signaling</td>
<td>5</td>
<td>APCS, C4BP, FGA, ORM1, SAA1</td>
<td>1.53E-05</td>
</tr>
<tr>
<td>Clathrin-mediated Endocytosis Signaling</td>
<td>5</td>
<td>ACTA1, ACTB, APOB, APOC1, ORM1</td>
<td>2.61E-05</td>
</tr>
<tr>
<td>Granulocyte Adhesion and Diapedesis</td>
<td>4</td>
<td>ACTA1, ACTB, MMP1, MMP3</td>
<td>3.53E-04</td>
</tr>
<tr>
<td>Production of Nitric Oxide and Reactive Oxygen Species in Macrophages</td>
<td>4</td>
<td>APOB, APOC1, MPO, ORM1</td>
<td>3.09E-04</td>
</tr>
</tbody>
</table>
Figure 2.2 Cellular localization of the 44 upregulated proteins based on GO annotation.

The numbers depicted represent the number of proteins with the specified cellular localization.

Table 2.3 Tissue expression of the top 20 elevated proteins identified from SF

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Human Protein Atlas</th>
<th>BioGPS*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tissue Expression</td>
<td>Tissue Expression</td>
</tr>
<tr>
<td>C4BP</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>APCS</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>ORM1</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>CD5L</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>M2BP</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>PLS2</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>MPO</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>SERPINB1</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>MMP1</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>APOC1</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>CTSG</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>BASP1</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>H4</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>PFN1</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>H2AFX</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>MMP3</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>SAA1</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>DEFA1</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>CRP</td>
<td>Skin</td>
<td>Immune</td>
</tr>
<tr>
<td>S100A9</td>
<td>Skin</td>
<td>Immune</td>
</tr>
</tbody>
</table>

*BioGPS does not contain expression data for bone tissue
2.3.2 SRM Verification of putative markers in individual SF samples

To verify the differences in protein expression between PsA and early OA SF samples, as identified by LC-MS/MS, we developed multiplexed selected reaction monitoring assays. Reactions were developed for 17 peptides representative of the 17 proteins with increased expression in the SF of PsA patients, as well as 4 peptides representing the 2 housekeeping proteins that had unchanged expression between PsA and OA SF (Table 2.4). Consistent with
our LC-MS/MS analysis of the pooled samples, overexpression of 13 out of these 17 proteins was also verified in the individual PsA SF samples (Set I), as compared to the early OA SF (Figure 2.3). Each sample consisted of two technical replicates. CRP, MMP3, and S100A9, were amongst these 13 verified proteins. The mean fold change of each protein and the associated P-values are depicted in Table 2.5.
Figure 2.3 Verification of elevated proteins in PsA synovial fluid (Set I) by selected reaction monitoring assays, normalized against housekeeping protein.

Dots represent SF samples from individual subjects; thin horizontal lines depict the mean, and vertical lines the SD. **** indicates P<0.0001; ***P<0.001; **P<0.01; *P<0.05; ns: non-significant.
Additionally, we also confirmed the elevation of 12 out of the 17 proteins, in an independent set of 10 PsA, and 10 early OA SF samples (Set II). In this case, we utilized heavy-labeled peptides in order to obtain an absolute concentration of these peptides in SF (Figure 2.4). The proteins included MPO, M2BP, DEFA1, H4, H2AFX, ORM1, CD5L, PFN1, and C4BP, as well as our positive controls MMP3, S100A9, and CRP. The mean fold change and P-values corresponding to each protein from SF set II are also depicted in Table 2.5.

Table 2.5 Summary of the fold change (FC) of candidate markers in SF Set I and II*

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Set I PsA:OA FC</th>
<th>P-Value</th>
<th>Set II PsA:OA FC</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORM1</td>
<td>2.1</td>
<td>0.0217</td>
<td>1.6</td>
<td>0.0487</td>
</tr>
<tr>
<td>CTSG</td>
<td>2.6</td>
<td>0.0001</td>
<td>2.4</td>
<td>0.0887</td>
</tr>
<tr>
<td>PFN1</td>
<td>2.6</td>
<td>0.0006</td>
<td>1.9</td>
<td>0.0299</td>
</tr>
<tr>
<td>H4</td>
<td>2.2</td>
<td>0.0271</td>
<td>2.5</td>
<td>0.0015</td>
</tr>
<tr>
<td>H2AFX</td>
<td>2.2</td>
<td>0.0022</td>
<td>3.3</td>
<td>0.0092</td>
</tr>
<tr>
<td>BASP1</td>
<td>1.9</td>
<td>0.8115</td>
<td>2.9</td>
<td>0.1220</td>
</tr>
<tr>
<td>MMP1</td>
<td>1.9</td>
<td>0.5580</td>
<td>1.5</td>
<td>0.6182</td>
</tr>
<tr>
<td>SERP1N1B1</td>
<td>2.0</td>
<td>0.4623</td>
<td>1.4</td>
<td>0.3298</td>
</tr>
<tr>
<td>MPO</td>
<td>3.5</td>
<td>0.0001</td>
<td>2.8</td>
<td>0.0039</td>
</tr>
<tr>
<td>PLS2</td>
<td>2.0</td>
<td>0.3051</td>
<td>1.0</td>
<td>0.9365</td>
</tr>
<tr>
<td>M2BP</td>
<td>2.3</td>
<td>0.0041</td>
<td>3.0</td>
<td>0.0048</td>
</tr>
<tr>
<td>C4BP</td>
<td>2.3</td>
<td>0.0016</td>
<td>2.1</td>
<td>0.0105</td>
</tr>
<tr>
<td>CRP</td>
<td>2.9</td>
<td>0.0001</td>
<td>2.8</td>
<td>0.0010</td>
</tr>
<tr>
<td>S100A9</td>
<td>2.8</td>
<td>0.0001</td>
<td>3.9</td>
<td>0.0010</td>
</tr>
<tr>
<td>MMP3</td>
<td>2.9</td>
<td>0.0001</td>
<td>3.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>DEFA1</td>
<td>2.1</td>
<td>0.0086</td>
<td>2.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>CD5L</td>
<td>2.1</td>
<td>0.0005</td>
<td>2.5</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

*Set I and Set II are described in the experimental methods section
Figure 2.4 Verification and concentration of elevated proteins in PsA synovial fluid (Set II) by selected reaction monitoring assays, normalized against heavy-labeled peptides.

Dots represent SF samples from individual subjects; thin horizontal lines depict the mean, and vertical lines the SD. **** indicates P<0.0001; ***P<0.001; **P<0.01; *P<0.05; ns: non-significant.
2.4 Discussion

In the present study, we performed proteomic analysis of SF, and identified 137 proteins that were differentially expressed between PsA and OA SF. Since one of our future goals is to investigate these proteins as serum biomarkers, we chose to focus only on the 44 upregulated proteins. Moreover, these proteins were reduced to 17 promising candidates that are likely produced by the synovial membrane, cartilage, or surrounding inflammatory cells, and are secreted into the synovial fluid compartment. The elevation of 12 of these proteins was confirmed in an independent SF cohort, using SRM assays, in order to provide sensitive and specific quantification. As an internal validation of our approach, we re-discovered a number of proteins previously known to be involved in the context of PsA or psoriasis progression. For example, our top 3 up-regulated proteins (based on fold change) - S100A9 (168, 169), MMP3, and CRP (115, 167) - have been extensively studied in the context of PsA.

Psoriasis is a T-cell driven disease that causes epidermal hyperplasia, with an influx of autoimmune effector cells including monocytes, neutrophils, and dendritic cells (1). Erythema is also an important feature of psoriasis and is caused by increased growth and dilation of superficial blood vessels in the skin (1). Joint and synovial findings in PsA are T-cell driven, as in the skin, and the prominent hyperplasia of blood vessels in skin is echoed in the synovial membrane (148). PsA also causes hyper proliferation of the lining cells of the synovium, resulting in the formation of invasive, inflammatory tissue (150, 170, 171). These aspects are reflected in the current study, through the identification of upregulated proteins belonging to I) acute-phase response signalling, II) granulocyte adhesion and diapedesis, III) production of
nitric oxide and reactive oxygen species in macrophage pathways, and IV) cell-to-cell signalling and interaction, cell movement, and antigen presentation.

One of these proteins, alpha defensin 1 (DEFA1), is secreted by neutrophils in response to various antigens. Elevated levels have been associated with inflammatory activity in both rheumatoid arthritis (172) and psoriasis (173). Additionally, antimicrobial defense mechanisms mediated by DEFA1 have also been described in inflammatory synovium (174). Although a minor inflammatory process is present in OA synovial tissue, PsA patients demonstrate a more aggressive state of synovial tissue inflammation compared with OA patients, therefore elevation in DEFA1 may be a good indicator of PsA pathogenesis.

The enzyme myeloperoxidase (MPO) also seems to play a role in the joint inflammation associated with PsA, as we have determined that it is elevated in PsA SF. MPO is the predominant protein present in primary granules of circulating polymorphonuclear cells. It is a member of the human peroxidase family haeme-containing enzymes that play a role in host defence against infection, and is the major enzymatic source of leukocyte-generated oxidants, released by activated neutrophils and used as a marker of leukocyte recruitment and function and subsequent inflammation (175). Although not specifically in PsA, MPO has been shown to be elevated in SF and serum derived from RA patients (175) and has been linked to the maintenance of oxidative stress in both psoriasis (176) and RA (177). Biological similarities between PsA and RA have been previously described, especially among PsA patients with polyarticular peripheral arthritis (150, 171). Increased expression of MPO may also represent an important mediator of PsA. Furthermore, CD5-like protein (CD5L) has been described as an alternative ligand for CD5, a lymphoid-specific membrane glycoprotein that is constitutively
expressed in all T cells, but expressed in highest levels in activated T-cells (178). Recently, circulating CD5 levels were shown to be increased in RA and systemic lupus erythematosus (179), and currently we have confirmed the elevation of its ligand, CD5L, in PsA SF. Although the functional relevance of CD5L is unknown, it may play a role in the stimulation and regulation of the immune system (179). Moreover, a set of recent experiments have provided evidence showing that CD5 stimulation, favors Th17- over Th1-driven polarization of naïve T-cells (180); the functional relevance of CD5L in this has not been investigated yet. This is relevant since in both psoriatic skin and synovium, while CD8+ T-lymphocytes predominate, the important role played by the Th17 subset of CD4+ T-cells in psoriatic disease has recently also become apparent (57-59, 181). Together, these findings corroborate our hypothesis, that the proteins we have identified are relevant to PsA and the underlying mechanisms that potentiate this disease.

We also uncovered novel putative mediators, which have yet to be described in arthritides, which include BASP1, H2AFX, and ORM1. Interestingly, of these, only ORM1 has been previously associated with psoriasis, where ORM1 was increased in the plasma of psoriatic patients. The specific function of this protein has not been determined yet, although it is believed to be involved in aspects of immunosuppression (182). We speculate that ORM1 may have a protective role in suppressing the immune system to decrease inflammation.

Despite these interesting findings, there are some aspects in the design of this study that need to be further clarified. First, the “control” synovial fluid originated from joints of patients with early OA, and not patients with psoriasis without PsA. We decided to use these samples since
SF from psoriasis patients is difficult to obtain. Moreover, it has been shown that early OA represents an appropriate comparison, to our inflammatory PsA SF (147).

Second, pooling of samples in the discovery phase of a proteomic study could potentially mask meaningful discrepancies among the different individual SF proteomes. While pooling of biological replicates does not allow for statistical comparison, it does produce sufficient sample and increases the likelihood of identifying proteins that are otherwise undetectable (low abundance) in individual samples, therefore allowing a more extensive proteomic coverage of the disease’s heterogeneity. In the current study, we pooled biological replicates and performed SCX fractionation of each pool in triplicate. With the discovery step, we intended to generate a comprehensive SF dataset to identify potential mediators and select candidates for SRM. Therefore, by pooling samples and subsequently quantifying proteins in individual samples, we have taken advantage of two complementary approaches in order to obtain more meaningful results.

Third, many groups have previously utilized albumin depletion prior to in-gel separation (97), and subsequent proteomic analysis, in order to simplify the SF protein content, and reduce the dynamic range of proteins. We chose to omit this step for the following reasons: I) albumin is a fundamental carrier protein, and therefore, its depletion would result in loss of potentially important proteins from our analysis (97), and II) including a step of immune depletion to our analysis has the potential of increasing the percent error and reducing the reproducibility of our experiments, both of which could increase our false discovery rate (133). In fact, in a pilot study where we assessed the protein recovery following albumin depletion, we determined that our protein recovery was 20-40% lower (data not shown).
As discussed previously, quantification of protein levels has been a major challenge in proteomics (136). We utilized a label-free approach in order to quantify, and identify upregulated proteins in PsA SF. In general, the advantage of label-free approaches over chemical labeling lies in the low cost and the high number of samples that can be easily included in the experiment. Potential disadvantages are lower reproducibility, which may compromise detection of smaller quantitative changes between samples. The lower reproducibility mostly results from the fractionation of peptides prior to LC-MS/MS analysis, and the subsequent pooling of eluted fractions, which can result in unequal/uncontrolled pooling, as was the case in the present study. For example, based on our SCX chromatographic spectra, we noticed several PsA fractions contained higher protein amounts, and pooling of these fractions could hinder the identification of low-abundance proteins; the high-protein fractions were therefore analyzed individually in the PsA group, resulting in the higher number of SCX fractions (15, compared to 10 in early OA, as described in the Methods section). To ensure accuracy and reproducibility of the SCX chromatography, following LC-MS/MS analysis, several peptides were chosen at random, and their occurrence was monitored across fractions corresponding to the PsA and early OA pools. Although the peptide profile was not identical in each fraction, as several peptides in the PsA group spanned multiple fractions, generally the peptide having the highest abundance was found in the same fraction when comparing early OA and PsA groups. Additionally, the reproducibility of LFQ experiments is also largely based on the timeframe of the experiment (97); therefore, in the present study, we standardized the entire pipeline, from sample collection and processing, to instrument setup and calibration. Despite the shortcomings of the fractionation and pooling strategy, we did verify the
overexpression of particular proteins using specific SRM assays, which provides validity to our entire strategy.

Overall, this study represents the most comprehensive proteomic analysis of PsA synovial fluid, to date. We discovered and verified 12 proteins significantly elevated in PsA SF, compared to early OA SF. Interestingly, the majority of these proteins are part of functional pathways that are known to be dysregulated in psoriasis or PsA. These proteins may serve as potential mediators of the pathogenesis of PsA, and should be further investigated in functional experiments. Also, a large-scale validation of these proteins in serum is essential, in order to investigate these proteins as putative biomarkers and/or therapeutic targets for the detection and treatment of PsA.
The data presented in this chapter has been published in Clinical Proteomics:

3 Quantitative proteomic analysis of skin biopsies from patients with psoriasis and psoriatic arthritis

3.1 Introduction

Psoriatic arthritis (PsA) is a distinct inflammatory arthritis, which takes its name from its association with the cutaneous, autoimmune inflammatory disease, psoriasis. It occurs in 30% of psoriasis patients, with a predicted prevalence of up to 1% in the general population. PsA is a complex, potentially disabling musculoskeletal disorder often arising early in age. Patients with PsA have an increased risk for a spectrum of co-morbidities, such as obesity, metabolic syndrome, diabetes, and cardiovascular disease (31, 32). The diagnosis of PsA presents a challenge, largely due to its heterogeneous clinical presentation (80, 81); however, early diagnosis of PsA is essential for prevention of joint damage and disability (88, 89).

The key to early diagnosis is a better recognition of PsA in patients with psoriasis, since the presence of psoriasis indicates a high risk of the presence or future development of PsA (31). Soluble biomarkers represent the ideal means for screening patients for PsA. With improvements in high-throughput genomic platforms, a number of putative markers, ranging from susceptibility genes, to mRNA profiles have been proposed (9, 41, 42, 106, 151, 152, 183-185) however there exists no single (or panel) of specific markers, or mediating factor(s). Much of the research, therefore, focuses on the discovery and validation of PsA biomarkers (186-189). Identifying these factors would not only facilitate diagnosis and prognosis of PsA, but provide further insight into disease pathogenesis. Mass spectrometry (MS)-based quantitative proteomic approaches are well-suited for the discovery of protein mediators and biomarkers of disease.
Specifically, label-free quantification methods have recently been optimized, where quantification is based on the differential peak intensity of the peptides in each MS scan (125, 157). Such experiments often result in tens to hundreds of candidate biomarkers that must be subsequently verified in serum (97). Therefore, in this context of biomarker discovery, high-throughput proteomics using mass spectrometry is a powerful tool for obtaining disease-specific proteome profiles of virtually any biological material. Human plasma represents a diverse proteome and is an excellent source of protein mediators of disease, but proteins secreted by tissues are diluted in blood, and are often undetectable by current MS methods. Much interest has been given to the analysis of proximal fluids and tissues, such as synovial fluid and skin. These represent logical compartments for PsA biomarkers since they are derived directly from the diseased site and function in the exchange of proteins between the affected site and the systemic circulation.

Cutaneous psoriasis develops simultaneously or precedes the onset of PsA in up to 90% of PsA patients, and in order to move forward in the search for PsA screening biomarkers, we must consider the preceding cutaneous psoriasis stage in PsA patients. Therefore, comparative analysis of skin between patients with PsA and those with psoriasis but without PsA (PsC) represents a reasonable experimental workflow. In a pilot study, our group demonstrated that proteins elevated in the inflamed skin, are likewise upregulated at the serum level, and may serve as putative markers of PsA (160).

In the current study, we performed label-free quantitation of skin proteins from PsA and PsC patients. Using selected reaction monitoring assays (SRM) we confirmed the elevation of some proteins in an independent set of samples from patients with PsA. Following a small-scale
validation in serum using ELISAs, we confirmed a significant elevation of β5 Integrin (ITGB5) in the serum of PsA patients. Periostin (POSTN) also showed a trend towards association. These proteins may not only serve as potential PsA biomarkers, but may also shed new light into the pathogenesis of PsA.
3.2 Methods

3.2.1 Skin proteomic analysis

3.2.1.1 Clinical samples

Samples were collected with informed consent after institutional review board approval was obtained from the University Health Network. For the discovery phase, skin biopsies were obtained from 10 PsA (6 males, 4 females; age range 38-73) and 10 PsC patients (6 males, 4 females; age range 28-77 years) (Set I). PsA patients had psoriasis and satisfied the CASPAR classification criteria (27), while the PsC patients were assessed by a rheumatologist, to exclude arthritis. Patients were not undergoing treatment with methotrexate (MTX) or anti-TNF agents. One 6mm punch biopsy was obtained from unaffected (non-lesional) skin, and one from affected (lesional) skin from each PsA and PsC patient, amounting to a total of 40 samples.

For the verification (quantification) phase, an independent set of skin samples was acquired from 5 PsA patients (all males; age range 49-63 years), and 5 PsC patients (3 males, 2 females; age range 49-67) (Set II). Inclusion criteria were the same as described above.

For the small-scale validation, serum samples were obtained from 33 PsA patients (22 males, 11 females; age range 21-76), and 15 PsC controls (9 males, 6 females, age range 28-77). Inclusion criteria were the same as described above.
3.2.1.2 Pre-analytical sample processing

Skin samples were snap-frozen in liquid nitrogen and stored at -80°C until use. Samples were suspended in 0.05% RapiGest buffer (Waters, Milford, MA, USA), and the tissue was homogenized and sonicated for protein extraction. The tissue lysates were spun at 11,000g and total protein was measured by the Bradford assay (Pierce Biotechnology, Rockford, IL, USA), on the resulting supernatants. Equal protein amounts from each sample were pooled to create eight different pools of five samples each [2 PsA lesional (PsA L), 2 PsA non-lesional (PsA N), 2 PsC lesional (PsC L), and 2 PsC non-lesional (PsC N)]. Proteins in each pool were denatured and reduced with 5 mM dithiothreitol at 60°C for 45 minutes and alkylated with 15 mM iodoacetamide, in the dark at room temperature for 45 minutes. Sequencing grade trypsin (Promega, Madison, WI, USA) was added in a 1:50 (trypsin: protein) ratio, and allowed to digest for 18 hours at 37°C. Trifluoroacetic acid (1%) was added to cleave RapiGest and inhibit trypsin activity, and samples were centrifuged at 11,000g for 20 minutes prior to high performance liquid chromatography (HPLC) using strong cation exchange (SCX) to reduce peptide mixture complexity.

3.2.1.3 HPLC-SCX

Digested samples were diluted 1:2 in mobile phase A SCX buffer (0.26 M formic acid (FA), 10% acetonitrile (ACN); pH 2.5) and loaded directly onto a 500 μL loop connected to a PolySULFOETHYL A column (2.1 mm inside diameter × 200 mm length; 5 μm particle size; 200 Å pore size; The Nest Group Inc., MA, USA), containing a silica-based hydrophilic, anionic polymer (poly-2-sulfoethyl aspartamide). An Agilent 1100 HPLC system was used for
fractionation. A 60 minute gradient was employed with a linear gradient starting at 30 minutes and consisting of mobile phase A and mobile phase B (0.26 M FA, 10% ACN, 1 M ammonium formate; pH-4.5) for elution of peptides (flow rate 200uL/min). The fractionation was monitored at a wavelength of 280 nm and performed in duplicate. Fractions were collected every two minutes from 20 to 55 minutes, and those with a low peak absorbance were pooled, resulting in a total of 12 fractions per sample. This amounted to a total of 96 SCX fractions, which were then subjected to liquid chromatographic and tandem mass spectrometric analysis (LC-MS/MS). SCX column and system performance was ensured by running a quality control peptide mixture consisting of 1ug/uL Alpha Bag Cell peptide, 1ug/uL Fibrinogen fragment, 5ug/uL Human ACTH, and 5ug/uL ACE Inhibitor (American Protein Company, CA, USA) after every sample.

3.2.1.4 LC-MS/MS

The SCX fractions were purified through C-18 OMIX Pipette Tips (Agilent Technologies, Germany), to remove impurities and salts, and eluted in 5 μL of 65% MS buffer B (90% ACN, 0.1% FA, 10% water, 0.02% trifluoroacetic Acid (TFA)) and 35% MS buffer A (5% ACN, 0.1% FA, 95% water, 0.02% TFA). The samples were diluted to 85 μL in MS buffer A and injected into a nano-LC system (Proxeon Biosystems, FL, USA) connected online to an LTQ-Orbitrap (Thermo Fisher Scientific, USA). A 90 minute linear gradient reversed phase chromatography using MS buffer A and MS buffer B, was performed at a flow rate of 400 nL/min to resolve peptides on a C-18 column (75uM x 5 cm; Proxeon Biosystems, FL). The MS parameters were: 300 Da minimum mass, 4000 Da maximum mass, automatic precursor charge selection, 10 minimum peaks per MS/MS scan; and 1 minimum scan per group. XCalibur software v.2.0.7 (Thermo Fisher Scientific, USA) was used for data acquisition.
3.2.1.5 Protein identification and quantification

Raw files corresponding to PsA L, PsA N, PsC L, and PsC N data sets were uploaded into MaxQuant v.1.2.2.2 (www.maxquant.org) (139) and searched with Andromeda (built into MaxQuant) (138) against the non-redundant IPI.Human v.3.71 database (86, 309 sequences; released March 2010). Search parameters included a fixed carbamidomethylation of cysteines and variable modifications of methionine oxidation and N-terminal acetylation. Data was initially searched against a “human first search” database with a parent tolerance of 20 ppm and a fragment tolerance of 0.5 Da in order to calculate and adjust the parent tolerance to 5 ppm for the search against the IPI.Human fasta file. During the search, the IPI.Human fasta database was randomized and the false discovery rate was set to 1% at the peptide and protein levels. Data was analyzed using “Label-free quantification” checked, and the “Match between runs” interval was set to 2 min. Proteins were identified with a minimum of one unique peptide. “LFQ Intensity” columns corresponding to the extracted ion current (XIC) value of each protein in replicate PsA L, PsA N, PsC L, and PsC N were averaged, and used to calculate PsA/PsC lesional and non-lesional ratios (fold change; FC).

3.2.1.6 Bioinformatics analysis

To detect upregulated proteins in the PsA L, one-sided Student’s t-tests were used between PsA L and PsC L, and PsA N and PsC N after log transformation of XIC values. Only proteins displaying significant differences [False discovery rate (FDR) <0.2] were used for further analysis. Averages of the replicate XIC values were calculated for each PsA L, PsA N, PsC L, and PsC L, and ratios (FC) of PsA L/PsC L, and PsA N/PsC N were computed.
Gene names of the upregulated proteins were checked against gene [BioGPS (http://biogps.org/#goto=welcome)](162), and protein [Human Protein Atlas (http://www.proteinatlas.org/)](163) databases, to identify proteins with strong expression in PsA-associated tissues and cell types (skin, bone, immune cells).

The Plasma Proteome Database ([http://www.plasmaproteomedatabase.org/](126)) was employed to identify proteins present in high-abundance in serum, which could represent potential contaminants. Selected reaction monitoring assays were developed for the top upregulated proteins in the lesional PsA group and housekeeping (ACTB, TUBB) proteins, and relative protein quantification was performed in individual skin samples to confirm their elevation in PsA skin.

### 3.2.2 Verification of identified proteins using SRM

SRM methods were developed for verification of protein ratios in skin, as described in Chapter 2.

#### 3.2.2.1 SRM assay development

PeptideAtlas ([http://www.peptideatlas.org/](http://www.peptideatlas.org/)) was utilized to select 3–4 tryptic peptides for the proteins of interest. The uniqueness of peptides was verified by BLAST ([https://blast.ncbi.nlm.nih.gov/Blast.cgi](https://blast.ncbi.nlm.nih.gov/Blast.cgi)). To identify peptide fragments (transitions) to monitor, in silico peptide fragmentation was performed using Pinpoint software (Thermo Fisher Scientific, USA) and 5–6 transitions were selected for each peptide. For method optimization, digested pooled samples of PsA lesional skin, used in our LC-MS/MS analysis, were loaded onto a C-18 column (Proxeon Biosystems, FL, USA) coupled to a triple quadrupole mass
spectrometer (TSQ Vantage; Thermo Fisher Scientific, USA), and approximately 2500 transitions were monitored in 25 subsequent runs. Three transitions were used for subsequent quantification.

3.2.2.2 Skin sample preparation

Fifty µg of total protein of each skin sample were denatured by heat, reduced, alkylated, and trypsin-digested as described previously. In sample set II, 500 fmol of a heavy-labeled peptide (Thermo Fisher), were also added as an internal standard. The heavy peptide sequence was LGPLVEQGR, where the C-terminal arginine was labeled with $^{13}$C and $^{15}$N. The resulting peptides were purified through C-18 OMIX Pipette Tips (Agilent), and eluted in 3µL of 65% MS buffer B and 35% MS buffer A. Samples were diluted to 40µL of MS buffer A, randomized, and loaded onto a C-18 column coupled to a triple quadrupole mass spectrometer.

3.2.2.3 SRM Assays

SRM assays were developed on a triple-quadrupole mass spectrometer (TSQ Vantage) using a nanoelectrospray ionization source (nano-ESI, Proxeon Biosystems, FL), as previously described in Chapter 2. Briefly, a 60-minute, three-step gradient was used to load peptides onto the column via an EASY-nLC pump (Proxeon Biosystems, FL), and peptides were analyzed by a multiplexed SRM method. Quantification, in skin set I was performed after normalization against a set of 4 peptides corresponding to 2 housekeeping proteins, to offset technical variations. Each sample was analyzed in duplicate, using a 60-minute method, whereby 153 transitions were monitored. Quantification in SF set II was performed following normalization against the spiked-in heavy-labeled peptide, as described earlier. Each sample was analyzed in
duplicate, using a 60-minute method, whereby 156 transitions were monitored. Reproducibility of the SRM signal was confirmed by running a quality control solution of 0.1 fmol/μL BSA after every 10 runs.

3.2.2.4 SRM protein quantification

Raw files recorded for each sample were analyzed using Pinpoint software (Thermo Fisher) (144), and peptide XIC were extracted. Pinpoint was used for identification and visualization of transitions. In the first skin set, in order to control for technical variation between the samples, XIC corresponding to each endogenous peptide replicate were divided by the XIC corresponding to the average of the two housekeeping peptides. This value was then averaged amongst the two replicate runs, to obtain “Normalized XIC”. In skin set II, in order to control for technical variation between the samples and obtain a more robust quantitative value for the proteins of interest, the XIC value corresponding to each endogenous peptide, was normalized to the XIC value corresponding to the spiked-in heavy peptide, in order to obtain a light:heavy (L:H) ratio. Since we added a known amount of heavy peptide to our samples prior to analysis, we used the L:H ratio to calculate the relative concentration of each endogenous peptide corresponding to the proteins of interest.

3.2.3 Validation of verified proteins

3.2.3.1 Enzyme-linked immunosorbent assays

The concentration of ITGB5 in serum was measured using an in-house developed protocol. Sheep anti-human ITGB5 polyclonal antibody (R&D Systems, Minneapolis MN, USA; Cat.#
AF3824) was immobilized in a 96-well clear polystyrene plate by incubating 100 μL of 0.75 ng/μL capture antibody in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) overnight. The plates were washed three times with wash buffer (5 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween® 20, pH 7.8), after which the plate was blocked by adding 300 μL of 1% BSA in PBS to each well and incubated with shaking at room temperature for 60 min. The plates were then washed three times with wash buffer and incubated with 100 μL per well of ITGB5 recombinant protein standards (R&D Systems), or serum samples with shaking at room temperature for 2 h. ITGB5 standards and serum samples were diluted in 1% BSA in PBS, with all serum samples diluted 10-fold. After incubation, the plates were washed three times with wash buffer and incubated with 100 μL per well of biotinylated sheep anti-human ITGB5 detection antibody (R&D Systems; Cat.# BAF3824) (0.25ng/μL detection antibody in 1% BSA in PBS) with shaking at room temperature for 2 h. After washing the plates three times with wash buffer, 100 μL of streptavidin-conjugated horseradish peroxidase solution (diluted 200-fold in 1% BSA in PBS) was added to each well and incubated for 15 min with shaking at room temperature. A final wash of three times with washing buffer was followed by the addition of 100 μL of 3,3’,5,5’-Tetramethylbenzidine (Sigma Aldrich, St, Louis, MO, USA) per well and incubated with shaking at room temperature for 10 min. The chromogenic reaction was stopped with the addition of 50 μL of 2 mol/L hydrochloric acid solution per well. Subsequently, the absorbance of each well was measured with the Wallac Envision 2103 Multilabel Reader (Perkin Elmer, MA) at 450 nm, standardized to background absorbance at 540 nm. Final serum concentrations were calculated by multiplying by the dilution factor.
The concentration of POSTN in serum was measured using a custom multiplexed Luminex Screening Assay (R&D Systems; Cat.# LXSAH), according to manufacturer’s instructions. Serum was diluted 100-fold.

3.2.3.2 Statistical analysis

The Student’s t-tests were performed to analyze the LC-MS/MS data using R statistical software (http://www.r-project.org/). The remaining statistical analyses were performed using Graph Pad Prism v.6.0 for Mac (GraphPad Software, CA, USA). Results were analyzed using the nonparametric Mann-Whitney U test. P-values less than 0.05 were considered statistically significant. Spearman’s rank correlation coefficient was used to assess the correlations between serum levels of POSTN, and ITGB5. Concentrations are reported in the text as mean ± standard deviation (SD).
Figure 3.1 Summary of the biomarker discovery, verification, and preliminary validation experimental workflow in skin
3.3 Results

3.3.1 Delineating the PsA skin proteome

Our LC-MS/MS analysis yielded a list of 1922 quantifiable proteins. Student’s t-tests were utilized to identify upregulated proteins between the PsA and PsC lesional groups, and this yielded a total of 62 proteins [false discovery rate (FDR) <0.2]. Generally, these proteins exhibited a ratio above 4.0. In parallel, a comparison between PsA and PsC non-lesional skin, demonstrated elevated expression of 131 proteins (FDR<0.2). In comparing the proteins identified from the two independent analyses (PsA L vs PsC L, and PsA N vs. PsC N), only 7 of these proteins are common.

Since our scope was to identify proteins elevated in lesional PsA skin, we decided to focus on the 62 proteins overexpressed in the PsA lesional skin, since they are most likely to represent mediators and potential biomarkers of PsA. We further focused on proteins displaying strong expression in skin, bone, and immune regulatory cells, but excluded immunoglobulins, unknown proteins, and high abundance serum proteins from further analysis. Table 3.1 presents the final list of 47 proteins, the corresponding fold change (FC) between PsA and PsC samples, and the P-Value and FDR associated with each protein. These were then assessed and quantified, using a multiplexed selected reaction monitoring assay, in individual skin samples pertaining to the discovery set (Set I), as well as in an independent set of 10 samples (Set II).
Table 3.1 Fold change (FC) of 47 elevated proteins identified by LC-MS/MS in PsA skin when compared to PsC skin

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Gene Name</th>
<th>PsA L :PsC L FC</th>
<th>P-Value***</th>
<th>FDR****</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periostin Osteoblast Specific Factor</td>
<td>POSTN</td>
<td>2.3</td>
<td>0.006</td>
<td>0.18</td>
</tr>
<tr>
<td>Adenylate Kinase 1</td>
<td>AK1</td>
<td>2.4</td>
<td>0.004</td>
<td>0.16</td>
</tr>
<tr>
<td>Heat Shock 70kDa Protein 5</td>
<td>HSPA5</td>
<td>2.5</td>
<td>0.008</td>
<td>0.19</td>
</tr>
<tr>
<td>WNT1 inducible signaling pathway protein 2</td>
<td>WISP2</td>
<td>2.5</td>
<td>0.002</td>
<td>0.15</td>
</tr>
<tr>
<td>Eukaryotic Translation Initiation Factor 2A</td>
<td>EIF2A</td>
<td>2.9</td>
<td>0.007</td>
<td>0.18</td>
</tr>
<tr>
<td>Activator Of Multicatalytic Protease Subunit 3</td>
<td>PSME3</td>
<td>3.2</td>
<td>0.003</td>
<td>0.16</td>
</tr>
<tr>
<td>Alpha-crystallin B chain</td>
<td>CRYAB</td>
<td>3.9</td>
<td>0.006</td>
<td>0.18</td>
</tr>
<tr>
<td>Proteasome 26S Subunit ATPase 2</td>
<td>PSMC2</td>
<td>3.9</td>
<td>0.001</td>
<td>0.12</td>
</tr>
<tr>
<td>Platelet-activating factor acetylhydrolase IB</td>
<td>PAFAHB2</td>
<td>4.5</td>
<td>0</td>
<td>0.12</td>
</tr>
<tr>
<td>Keratin 18</td>
<td>KRT18</td>
<td>4.6</td>
<td>0.006</td>
<td>0.18</td>
</tr>
<tr>
<td>Carboxypeptidase N 2</td>
<td>CPN2</td>
<td>5.7</td>
<td>0.002</td>
<td>0.15</td>
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<tr>
<td>60S ribosomal protein L19</td>
<td>RPL19</td>
<td>6.6</td>
<td>0.001</td>
<td>0.12</td>
</tr>
<tr>
<td>Signal Recognition Particle</td>
<td>SRP14</td>
<td>6.8</td>
<td>0.003</td>
<td>0.16</td>
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<tr>
<td>Four And A Half LIM Domains 1</td>
<td>FHL1</td>
<td>7</td>
<td>0.006</td>
<td>0.18</td>
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<tr>
<td>Proline Glutamine-Rich Splicing Factor</td>
<td>SFPQ</td>
<td>7.1</td>
<td>0.007</td>
<td>0.18</td>
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<tr>
<td>Asporin</td>
<td>ASPN</td>
<td>8.9</td>
<td>0.007</td>
<td>0.18</td>
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<tr>
<td>Gamma-Glutamyltransferase 5</td>
<td>GGT5</td>
<td>9</td>
<td>0.006</td>
<td>0.18</td>
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<tr>
<td>Cytochrome B5 Reductase 2</td>
<td>CYB5R2</td>
<td>9.1</td>
<td>0.006</td>
<td>0.18</td>
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<tr>
<td>Cytoplasmic FMR1 Interacting Protein 1</td>
<td>CYFIP1</td>
<td>10</td>
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<td>0.16</td>
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<tr>
<td>Ring Finger Protein 113A</td>
<td>RNF113A</td>
<td>10.4</td>
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<td>Vayl-TRNA Synthetase</td>
<td>VARS</td>
<td>11.7</td>
<td>0.005</td>
<td>0.18</td>
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<td>Hydroxysteroid (17-beta) dehydrogenase 4</td>
<td>HSD17B4</td>
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<td>0.003</td>
<td>0.16</td>
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<tr>
<td>Alpha Synuclein</td>
<td>SNCA</td>
<td>N/A**</td>
<td>0.003</td>
<td>0.16</td>
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<tr>
<td>Heat Shock 70kDa Protein 4-Like</td>
<td>HSPA4L</td>
<td>N/A</td>
<td>0.003</td>
<td>0.16</td>
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<tr>
<td>26S Proteasome Regulatory Subunit P40.5</td>
<td>PSMD13</td>
<td>N/A</td>
<td>0.004</td>
<td>0.17</td>
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<tr>
<td>KDEL Motif-Containing Protein 2</td>
<td>KDELC2</td>
<td>N/A</td>
<td>0.004</td>
<td>0.17</td>
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<tr>
<td>DNA Polymerase II Subunit A</td>
<td>POLE</td>
<td>N/A</td>
<td>0.005</td>
<td>0.18</td>
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<tr>
<td>Beta Integrin 5</td>
<td>ITGB5</td>
<td>N/A</td>
<td>0.006</td>
<td>0.18</td>
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<tr>
<td>Armadillo Repeat Containing 1</td>
<td>ARMC1</td>
<td>N/A</td>
<td>0.007</td>
<td>0.18</td>
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<tr>
<td>Transmembrane Emp24 Protein Transport Domain 7</td>
<td>TMED7</td>
<td>N/A</td>
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<td>Complement C1q</td>
<td>CIQC</td>
<td>N/A</td>
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<tr>
<td>Isoform 1 of UPF0505 protein C16orf62</td>
<td>C16orf62</td>
<td>N/A</td>
<td>0</td>
<td>0.12</td>
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<tr>
<td>Sushi repeat-containing protein</td>
<td>SRPX</td>
<td>N/A</td>
<td>0.001</td>
<td>0.12</td>
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<tr>
<td>Mercaptopyruvate Sulfurtransferase</td>
<td>MPST</td>
<td>N/A</td>
<td>0.001</td>
<td>0.12</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Gene Symbol</td>
<td>Ratio (PsA L vs. PsC L)</td>
<td>FDR</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td>------------------------</td>
<td>------</td>
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<tr>
<td>Leucine zipper and CTNNBIP1 domain containing</td>
<td>LZIC</td>
<td>N/A</td>
<td>0.001 0.12</td>
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<tr>
<td>Mitogen-Responsive Phosphoprotein Disabled Homolog 2</td>
<td>DAB2</td>
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<td>Angiopoiatin-like 2</td>
<td>ANGPTL2</td>
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<td>Glia maturation factor gamma</td>
<td>GMFG</td>
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<td>0.001 0.12</td>
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<td>Renin binding protein</td>
<td>RENBP</td>
<td>N/A</td>
<td>0.001 0.12</td>
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<tr>
<td>Ubiquitin-conjugating enzyme E2L 6</td>
<td>UBE2L6</td>
<td>N/A</td>
<td>0.001 0.12</td>
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<tr>
<td>Protein phosphatase 2A activator, regulatory subunit 4</td>
<td>PPP2R4</td>
<td>N/A</td>
<td>0.002 0.15</td>
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<tr>
<td>Heat shock factor binding protein 1</td>
<td>HSBP1</td>
<td>N/A</td>
<td>0.002 0.15</td>
<td></td>
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<tr>
<td>Glyoxylate reductase/hydroxypyruvate reductase</td>
<td>GRHPR</td>
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<td>0.002 0.15</td>
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</tr>
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<td>Eukaryotic translation initiation factor 2B, subunit 4 delta</td>
<td>EIF2B4</td>
<td>N/A</td>
<td>0.003 0.16</td>
<td></td>
</tr>
<tr>
<td>Sorting nexin 1</td>
<td>SNX1</td>
<td>N/A</td>
<td>0.003 0.16</td>
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<tr>
<td>Acyl-CoA Oxidase 1</td>
<td>ACOX1</td>
<td>N/A</td>
<td>0.001 0.12</td>
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<tr>
<td>G protein pathway suppressor 1</td>
<td>GPS1</td>
<td>N/A</td>
<td>0.006 0.18</td>
<td></td>
</tr>
</tbody>
</table>

*FC represents the ratio of the mean XIC values of 10 skin samples per group (PsA L vs. PsC L)  
**N/A indicates that a ratio could not be compiled since the protein was absent in PsC skin  
***P-Values were calculated using the student's t-tests  
****FDR represents the false discovery rate of each protein
3.3.2 SRM verification of putative markers in individual skin samples

We developed a multiplexed SRM assay to verify the differences in protein expression between lesional PsA and PsC skin. Assays were developed for 47 peptides representative of the 47 proteins with increased expression in the skin of PsA patients, as well as 4 peptides representing 2 housekeeping proteins (ACTB, TUBB). Consistent with our LC-MS/MS analysis of the pooled samples, overexpression of 12 out of the 47 proteins was also verified in the individual PsA lesional skin samples (Set I), when compared to the lesional PsC samples. Each sample consisted of two technical replicates. The mean FC of each confirmed protein in the lesional and non-lesional PsA and PsC skin samples, as well as the associated P-values are depicted in Table 3.2. The proteins included C16ORF62, SNCA, LZIC, SRP14, ITGB5, POSTN, SRPX, FHL1, PPP2R4, CPN2, GPS1, and PAFAH1B2. The distribution of these, and the housekeeping proteins across the Set I skin samples, are represented in Figure 3.2.
Figure 3.2 Distribution of significant markers and housekeeping proteins across the Set I PsA and PsC skin samples

Dots represent skin samples from individual subjects; thin horizontal lines depict the mean, and vertical lines the SD. **** indicates P<0.0001; ***P<0.001; **P<0.01; *P<0.05; ns: non-significant.
Table 3.2 Summary of the fold change of candidate markers in skin Set I and Set II

The description of Set I and Set II are given in the experimental methods section. Fold change (FC) represents the ratio of means of 10 (Set I) or 5 (Set II) skin samples per group. Data are based on normalized XIC ratios, as described in the experimental methods section. PsA, psoriatic arthritis; PsC, cutaneous psoriasis

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Set I</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lesional</td>
<td>Non-lesional</td>
<td>Lesional</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PsA:PsC FC**</td>
<td>P-Value***</td>
<td>PsA:PsC FC</td>
<td>P-Value</td>
<td>PsA:PsC FC</td>
<td>P-Value</td>
</tr>
<tr>
<td>CPN2</td>
<td>17.4 &lt;0.001</td>
<td>2.4 0.030</td>
<td>1.9 0.032</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPS1</td>
<td>6.0 0.014</td>
<td>1.2 0.385</td>
<td>17.9 0.008</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16ORF62</td>
<td>6.0 &lt;0.001</td>
<td>5.3 0.007</td>
<td>3.9 0.667</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FHL1</td>
<td>4.6 &lt;0.001</td>
<td>3.1 0.021</td>
<td>2.2 0.016</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRPX</td>
<td>3.8 0.043</td>
<td>3.3 0.014</td>
<td>5.0 0.008</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SNCA</td>
<td>3.6 &lt;0.001</td>
<td>1.7 0.089</td>
<td>3.8 0.095</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>POSTN</td>
<td>3.5 0.001</td>
<td>2.8 0.013</td>
<td>7.5 0.032</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PAFAH1B2</td>
<td>3.3 0.004</td>
<td>2.0 0.104</td>
<td>0.8 0.667</td>
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<tr>
<td>SRP14</td>
<td>3.0 0.019</td>
<td>1.3 0.570</td>
<td>2.4 0.016</td>
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<tr>
<td>ITGB5</td>
<td>2.7 0.006</td>
<td>3.5 0.017</td>
<td>4.2 0.032</td>
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<td>PPP2R4</td>
<td>2.2 0.043</td>
<td>2.0 0.678</td>
<td>3.9 0.008</td>
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<td></td>
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</tbody>
</table>

*The description of Set I and Set II are given in the experimental methods section.
**Fold change (FC) represents the ratio of means of 10 (Set I) or 5 (Set II) skin samples per group. Data are based on normalized XIC ratios, as described in the experimental methods section. PsA, psoriatic arthritis; PsC, cutaneous psoriasis
***P-Values were calculated using the non-parametric Mann-Whitney U test.
Additionally, we further confirmed the elevation of 8 of the aforementioned 12 proteins, in an independent set of 5 PsA, and 5 PsC lesional skin samples (Set II). In this case, we utilized a heavy-labeled peptide in order to obtain the absolute concentration of these peptides in the skin. The proteins included SRP14, ITGB5, POSTN, SRPX, FHL1, PPP2R4, CPN2, and GPS1. The mean FC and P-values corresponding to each protein from SF set II are also depicted in Table 3.2. Figure 3.3 shows the distribution of the 8 proteins across the Set II skin samples, as well as of the housekeeping proteins. Since only 8 proteins were confirmed in the independent sample set, we decided to continue all further analyses using these potential markers.
Figure 3.3 Distribution of significant markers across the Set II PsA and PsC skin samples

Dots represent skin samples from individual subjects; thin horizontal lines depict the mean and vertical lines the SD. **** indicates P<0.0001; ***P<0.001; **P<0.01; *P<0.05; ns: non-significant.
3.3.3 Small-scale ELISA validation in serum

To validate the expression of these markers in serum of PsA patients, we measured the levels of ITGB5 and POSTN in the serum of 15 PsC and 33 PsA patients. We were unable to measure SRP14, SRPX, FHL1, PPP2R4, CPN2, and GPS1, due to unavailability of ELISA kits, antibodies, or protein standards. As shown in Figure 3.4, ITGB5 was significantly elevated in PsA, when compared to PsC patients (1.19±0.5 compared to 0.77±0.6; p<0.01). The concentration of POSTN was not significantly different between the two groups, but the mean level in PsA serum were numerically higher compared to PsC serum (17.71±6.4 compared to 14.53±6.2). The mean FC and distribution of POSTN and ITGB5 in the PsA and PsC serum, is represented in Figure 3.4.

Figure 3.4 Distribution of markers across PsA (n=33) and PsC (n=15) serum sets

![Graph showing distribution of markers across PsA and PsC serum sets](image)

Dots represent serum samples from individual subjects; thin horizontal lines depict the mean, and vertical lines the standard deviation; FC represents the ratio of the mean concentration values corresponding to 33 PsA and 15 PsC serum samples. ** indicates P<0.01; ns: non-significant. PsA, Psoriatic Arthritis; PsC, Cutaneous Psoriasis
3.3.4 Correlation amongst markers

Spearman’s rank correlation coefficient was used to assess the correlation amongst markers for the PsA, and PsC serum groups. ITGB5 correlated significantly with POSTN in PsC serum (Spearman $r = 0.637$, $P = 0.013$), and in PsA serum (Spearman $r = 0.433$, $P = 0.012$). Figure 3.5 displays the correlation between POSTN and ITGB5 in all samples (Spearman $r = 0.472$, $P < 0.001$).

**Figure 3.5.** Correlation between ITGB5 and POSTN across the PsA and PsC serum sets.
3.4 Discussion

Recent advances in mass spectrometry-based proteomics have facilitated the identification of putative novel biomarkers and mediators from a variety of tissues, and for various clinical applications (165, 190, 191). In this study, we conducted high-throughput quantitative proteomic analyses to identify differentially expressed proteins between skin derived from PsA and PsC patients. According to our filtering criteria, forty-seven proteins were elevated in the PsA group, when compared to the PsC group. Eight proteins were verified using a multiplexed selected reaction monitoring assay, in an independent skin sample set. None of these potential markers has been described before in the context of PsA or psoriasis. Since serum measurement of these proteins using SRMs is not efficient due to the high complexity of the fluid, we utilized available enzyme-linked immunosorbent assays to measure two biomarker candidates in serum (ITGB5, and POSTN).

Cumulative evidence strongly supports the involvement of Interleukin-23/IL-17 axis in the pathogenesis of PsA (57-59, 120), and a number of compounds that target components of these pathways have been recently used in PsA clinical trials (24, 60). IL-23 acts synergistically with IL-6 and TGF-β to promote rapid Th17 development and IL-17 release (121, 122), which, in turn, plays a central role in sustaining chronic inflammation (121). Both POSTN and ITGB5 have been implicated, in some manner with this pathway.

Periostin is a matricellular protein belonging to the fascilin family (192). It interacts with several integrin molecules on cell surfaces, one of which is α5β5 Integrin, and provides signals for tissue development and remodeling (193). More specifically, it is thought that POSTN interacts with α5β5 to induce pro-inflammatory cytokine production via the Akt/NF-κB signaling pathway.
In support of this, deficiency of POSTN or inhibition of α5 integrin (ITGA5) prevented development or progression of skin inflammation in an Atopic Dermatitis mouse model (193). Additionally, POSTN has been shown to also act on immune cells, leading to their enhanced transmigration, chemotaxis, and adhesion (195), all of which further implicate this molecule in inflammatory processes relevant to PsA.

Apart from acting as a POSTN ligand (along with ITGA5), ITGB5 has also been described in rheumatoid arthritis, where it serves as a ligand for Cyr61 (195). Cyr61 is a molecule secreted by fibroblast-like synoviocytes in the joint, and stimulates IL-6 production via ITGA5-ITGB5/Akt/NF-κB signaling pathway (196). As described earlier, IL-6 production acts with IL-23 and TGF-β to trigger Th17 differentiation and IL-17 production (121, 122) in inflammatory processes as they also occur in PsA. As a result, we believe POSTN and ITGB5 are attractive molecules to investigate further as PsA biomarkers.

Following a small-scale serum validation of POSTN and ITGB5, we determined that only ITGB5 was significantly elevated in PsA serum, although POSTN also showed a trend. Additionally, Spearman correlation showed that serum ITGB5 correlates well with POSTN (r = 0.472, p<0.001), which may indicate that these two markers may, in the future, be used as part of a panel of markers to screen for PsA in PsC patients. The work reported here has some limitations. First, pooling of the skin samples in the discovery phase has both benefits as well as drawbacks. While pooling allows a more extensive coverage of the disease’s heterogeneity by increasing the likelihood of identifying proteins that are otherwise undetectable in individual samples, it may also mask meaningful discrepancies among the different individual skin proteomes. To minimize this effect, samples were pooled to obtain two distinct pools in both,
PsA and PsC sample groups. Additionally, all pool-derived candidates were further examined in all individual samples using a targeted and more sensitive mass spectrometry technique. Thus, by pooling samples as well as verifying protein expression in individual skin samples, we have taken advantage of two complementary approaches in order to obtain more meaningful results.

Second, the levels of some of the proteins, notably CPN2, differed considerably between the two verification sets. This could be due to the fact that Set I samples were the original discovery samples, and thus the large difference seen in Set I verification is expected. Additionally, the difference observed could simply be due to chance, which outlines the need to perform verification in hundreds of samples in order to gauge the true distribution of the markers. Unfortunately, skin biopsies were hard to obtain, and thus we were limited to only 10 and 5 samples for Set I and Set II, respectively.

Third, the identified markers were only tested on a small number of serum samples (n=48), and only 20 of these were distinct from the Set I and Set II sample sets. Therefore, to assess their distribution in serum, these markers still need to be tested in a large-scale verification study. In addition, as discussed previously, only two of the existing eight proposed markers have been investigated in serum due to the lack of ELISAs or antibodies. Hence, strategies to enable measurement of the remainder of the markers will have to be employed in the future.

Taken together, our current observations are consistent with the notion that label-free quantitative LC-MS/MS can be utilized to quantify proteins in tissues, which are shed into the circulation and serve as serum markers of PsA. Further studies to validate these findings are underway, in a larger and independent serum cohort. Investigating these proposed markers
further, may not only result in the identification of PsA screening biomarkers, but may also uncover aspects of PsA pathobiology that are currently unknown
Chapter 4
4 Validation of three candidate PsA biomarkers in serum

4.1 Introduction

PsA is an inflammatory arthritis associated with the chronic inflammatory skin disease, psoriasis. The estimated population prevalence of PsA is 0.25%, and it affects approximately 30% of patients seen in dermatology clinics (92, 197, 198). PsA is classified according to the CASPAR classification criteria (27), which provide high specificity and sensitivity when applied by rheumatologists (80, 81).

Patients with PsA have progressive joint damage, reduced quality of life, work-related problems, and more comorbidities when compared to psoriasis patients (200). Additionally, disease severity at the time of presentation is a risk factor for mortality (201, 202). There is increasing evidence that early diagnosis and management of PsA leads to better patient outcomes, and the key to early diagnosis is better recognition of PsA in patients with psoriasis (79, 87-89).

The presence of cutaneous psoriasis indicates a high risk for developing PsA. Studies conducted in dermatology clinics have shown high prevalence of undiagnosed PsA in psoriasis patients (32, 72, 198, 199). This is due to the fact that identifying inflammatory musculoskeletal disease in patients by non-rheumatologists is difficult since the symptoms are non-specific, and acute-phase reactants may be normal (80). While there are clinical features that can be utilized to predict PsA, such as nail, scalp, intergluteal/perianal psoriasis, and psoriasis severity, identifying soluble biomarkers that can identify PsA in psoriasis patients, may help in early diagnosis, and subsequent prevention of disability and improvement in quality of life (198, 200, 92, 80, 79).
With the aim of identifying new PsA biomarkers, we previously performed proteomic analysis of synovial fluid and skin biopsies obtained from PsA patients, using LC-MS/MS, and identified 20 putative PsA biomarkers. Of these, the current study details the validation of six candidates, M2BP, MPO, ITGB5, CD5L, MMP3, and CRP. These were selected based on the availability of commercial ELISA kits or antibodies, as well as preliminary verification studies reported in a previous publication.
4.2 Methods

4.2.1 Clinical samples

Consent was obtained from all study subjects. Research ethics board approval was obtained from the University Health Network. Serum samples were obtained from 100 cases with PsA, 100 with PsC, and 100 healthy controls. Patients with PsA had psoriasis, satisfied the CASPAR classification criteria (27), and had active PsA, defined by at least 3 swollen and tender joints. PsC patients had chronic plaque psoriasis and were assessed by a rheumatologist to exclude PsA. Controls represented individuals who did not have psoriasis or inflammatory arthritis. Patients with PsA and PsC were group matched for age, sex, and psoriasis severity and duration. Controls were matched for age and sex. The demographics, disease characteristics, and biomarker levels of recruited patients are demonstrated in Table 4.1.
Table 4.1 Demographics, disease characteristics, and serum biomarker levels in studied cohorts

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PsA (n=100)</th>
<th>PsC (n=100)</th>
<th>Controls (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females, %</td>
<td>41</td>
<td>45</td>
<td>52</td>
</tr>
<tr>
<td>Age(^a), years</td>
<td>51 (12.1)</td>
<td>49.8 (12.6)</td>
<td>35.2 (10.7)</td>
</tr>
<tr>
<td>Psoriasis duration(^a), years</td>
<td>22.9 (12.5)</td>
<td>20.3 (13.9)</td>
<td>-</td>
</tr>
<tr>
<td>PsA duration(^a), years</td>
<td>14.6 (12)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Swollen joint count(^a)</td>
<td>3.2 (3.5)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tender joint count(^a)</td>
<td>6 (8.1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clinically damaged joint count(^a)</td>
<td>7.4 (11.1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PASI score(^a)</td>
<td>4.7 (6.4)</td>
<td>4 (3.4)</td>
<td>-</td>
</tr>
<tr>
<td>Patients with current nail involvement, %</td>
<td>74</td>
<td>51</td>
<td>-</td>
</tr>
<tr>
<td>Patients on systemic agents, %</td>
<td>35</td>
<td>8</td>
<td>-</td>
</tr>
</tbody>
</table>

Biomarkers

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>PsA (n=100)</th>
<th>PsC (n=100)</th>
<th>Controls (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5L(^a), ng/mL</td>
<td>192.8 (197.1)</td>
<td>166.9 (147.4)</td>
<td>89.0 (145.1)</td>
</tr>
<tr>
<td>ITGB5(^a), ng/mL</td>
<td>1.5 (2.6)</td>
<td>0.7 (0.4)</td>
<td>0.8 (1.4)</td>
</tr>
<tr>
<td>M2BP(^a), ng/mL</td>
<td>295.6 (50)</td>
<td>269.6 (27.7)</td>
<td>232.9 (64.2)</td>
</tr>
<tr>
<td>MMP3(^a), ng/mL</td>
<td>44.3 (57.3)</td>
<td>22.2 (15.6)</td>
<td>17 (12)</td>
</tr>
<tr>
<td>MPO(^a), ng/mL</td>
<td>418.1 (270.9)</td>
<td>402.5 (217.3)</td>
<td>232.8 (126.6)</td>
</tr>
<tr>
<td>CRP(^a), mg/L</td>
<td>5.9 (2.9)</td>
<td>4 (3.8)</td>
<td>2.3 (2.1)</td>
</tr>
</tbody>
</table>

\(^a\) Values indicate mean (standard deviation)

No patients were undergoing treatment with biologics at the time of serum collection. Three patients with PsC were on methotrexate (MTX), while 5 were on retinoids. Thirty-five patients with PsA were on at least one form of systemic therapy (10 MTX, 13 leflunomide, 10 sulfasalazine, 3 hydroxychloroquine, 1 azathioprine, 1 cyclosporine). Blood samples were drawn at the time of clinical assessment, processed, and serum aliquots were stored at -80 until biomarker measurement.
4.2.2 Measurement of CD5L, ITGB5, MPO, MMP3, CRP, and M2BP

4.2.2.1 ITGB5

ITGB5 was measured according to our in-house developed protocol, as described in Chapter 3. Briefly, sheep anti-human ITGB5 polyclonal antibody (R&D Systems, Minneapolis MN, USA) was immobilized in a 96-well clear polystyrene plate by incubating 100 μL of 0.75 ng/μL capture antibody in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) overnight. The plates were washed three times with wash buffer (5 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween® 20, pH 7.8), after which the plate was blocked by adding 300 μL of 1% BSA in PBS to each well and incubated with shaking at room temperature for 60 min. The plates were then washed three times with wash buffer and incubated with 100 μL per well of ITGB5 recombinant protein standards (R&D Systems), or serum samples with shaking at room temperature for 2 h. ITGB5 standards and serum samples were diluted in 1% BSA in PBS, with serum samples diluted 10-fold. After incubation, the plates were washed three times with wash buffer and incubated with 100 μL per well of biotinylated sheep anti-human ITGB5 detection antibody (R&D Systems) (0.25ng/μL detection antibody in 1% BSA in PBS) with shaking at room temperature for 2 h. After washing the plates three times with wash buffer, 100 μL of streptavidin-conjugated horseradish peroxidase solution (diluted 200-fold in 1% BSA in PBS) was added to each well and incubated for 15 min with shaking at room temperature. A final wash of three times with washing buffer was followed by the addition of 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma Aldrich, St, Louis, MO, USA) per well and incubated with shaking at room temperature for 10 min. The chromogenic reaction was
stopped with the addition of 50 μL of 2 mol/L hydrochloric acid solution per well.

Subsequently, the absorbance of each well was measured with the Wallac Envision 2103 Multilabel Reader (Perkin Elmer, MA) at 450 nm, standardized to background absorbance at 540 nm. Final serum concentrations were calculated by multiplying by the dilution factor.

4.2.2.2 CD5L

The plates were washed three times with wash buffer, after which the plate was blocked by adding 300 μL of 1% BSA in PBS to each well and incubated with shaking at room temperature for 60 min. The plates were then washed three times with wash buffer and incubated with 100 μL per well of CD5L recombinant protein standards (R&D Systems; Cat.# 2797-CL-050), or serum samples with shaking at room temperature for 2 h. CD5L standards and serum samples were diluted in 1% BSA in PBS, with all serum samples diluted 10-fold. After incubation, the plates were washed three times with wash buffer and incubated with 100 μL per well of biotinylated goat anti-human CD5L detection antibody (0.1 ng/μL detection antibody in 1% BSA in PBS) with shaking at room temperature for 2 h. After washing the plates three times with wash buffer, 100 μL of streptavidin-conjugated horseradish peroxidase solution (diluted 200-fold in 1% BSA in PBS) was added to each well and incubated for 15 min with shaking at room temperature. A final wash of three times with washing buffer was followed by the addition of 100 μL of TMB per well and incubated with shaking at room temperature for 10 min. The chromogenic reaction was stopped with the addition of 50 μL of 2 mol/L hydrochloric acid solution per well. Subsequently, the absorbance of each well was measured with the Wallac Envision 2103 Multilabel Reader (Perkin Elmer, MA) at 450 nm, standardized to background
absorbance at 540 nm. Final serum concentrations were calculated by multiplying by the dilution factor.

4.2.2.3 M2BP

Additionally, the concentration of M2BP was measured using a commercially available ELISA kit (R&D Systems; Cat.# DY2226) according to manufacturer’s instructions. Samples were diluted 250-fold.

4.2.2.4 CRP, MMP3, MPO

A commercially available customized multiplexed Luminex Screening Assay (R&D Systems; Cat.# LXSAH) was purchased for MPO, MMP3, and CRP, and measurement of these markers was performed according to manufacturer’s instructions. Samples were diluted 100-fold.

4.2.3 Statistical analysis

The levels of most of the proteins are highly skewed to the right, and thus, all statistical analyses were based on log2 transformed protein levels. The significance of each group (Control, PsC, PsA) was tested for each biomarker to determine whether there was a significant difference in protein levels for two group comparisons: PsA vs PsC, and PsA vs Control. Statistical tests were performed while controlling for significant covariates, which could include age, sex, psoriasis duration, sample storage duration, PASI score, or drug treatment.

Effects of markers on patients with psoriasis alone vs controls and patients with PsA vs controls were compared using polychotomous logistic regression, a regression technique useful for categorical responses with three or more categories. In this way, we were able to test whether
the associations between each marker and disease status (PsA or PsC) are the same between the two disease groups. Univariate, and multivariate logistic regression models were used to identify which biomarkers were independently associated with PsA, when compared to PsC. These models were fit with disease classification as the outcome using biomarkers as explanatory variables while controlling for sex and age. Receiver operating characteristic (ROC) curves were constructed to investigate how biomarker data can best be used to classify patients into disease groups. The accuracy of the classification of patients was summarized using the area under the ROC curve. Finally, to determine if there is significant correlation between biomarkers, the Spearman's rank correlation coefficients were computed.
4.3 Results

The distribution of markers across the three serum sets is demonstrated below.

Figure 4.1 Distribution of markers across control (n=100), PsA (n=100), and PsC (n=100) serum sets

Dots represent serum samples from individual subjects; thin horizontal lines depict the mean, and vertical lines the standard deviation; PsA, Psoriatic Arthritis; PsC, Cutaneous Psoriasis
Table 4.2 outlines the significantly different biomarker levels, when comparing PsA to PsC, or PsA to controls. Increased levels of ITGB5 \( (p=7.14E-06) \), CRP \( (p=1.90E-07) \), MMP3 \( (p=7.04E-06) \), and M2BP \( (p=8.28E-04) \) are present in patients with PsA, when compared to PsC.

Similarly, elevated ITGB5 \( (p=1.11E-11) \), CRP \( (p=2.90E-17) \), MMP3 \( (p=5.72E-06) \), M2BP \( (p=2.01E-13) \), CD5L \( (p=5.72E-08) \), and MPO \( (p=3.66E-05) \), are present in PsA patients when compared to controls.

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>PsA compared to PsC</th>
<th>PsA compared to controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITGB5, ng/mL</td>
<td>1.76 (1.38, 2.25)</td>
<td>2.40 (1.88, 3.06)</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>2.24 (1.66, 3.02)</td>
<td>3.90 (2.89, 5.25)</td>
</tr>
<tr>
<td>MMP3, ng/mL</td>
<td>1.54 (1.28, 1.86)</td>
<td>1.58 (1.30, 1.93)</td>
</tr>
<tr>
<td>M2BP, ng/mL</td>
<td>1.11 (1.04, 1.17)</td>
<td>1.27 (1.20, 1.35)</td>
</tr>
<tr>
<td>MPO, ng/mL</td>
<td>1.13 (0.93, 1.36)</td>
<td>1.53 (1.25, 1.87)</td>
</tr>
<tr>
<td>CD5L, ng/mL</td>
<td>1.17 (0.83, 1.64)</td>
<td>2.62 (1.86, 3.68)</td>
</tr>
</tbody>
</table>

The results of the polychotomous logistic regression analysis to identify biomarkers that have differential associations between the three cohorts (PsA, PsC, Controls) are given in Table 4.3. The polychotomous logistic regression allows us to simultaneously investigate the differences between the three groups using logistic regression. The homogeneity \( P \)-values indicate the differences of the biomarker level among the three groups (PsC, PsA, and controls) when modeling PsC and PsA separately while controlling for age, sex, and the other biomarkers investigated. Furthermore, the \( p \)-values associated with PsA or PsC indicate the significance of biomarkers’ associations with PsA or PsC when using healthy controls as reference. We show that ITGB5 \( (p=1.18E-05) \), CRP \( (p=1.40E-06) \) and to a lesser extent M2BP \( (p=1.97E-03) \), are biomarkers that can distinguish between PsA from PsC. We also show that CD5L \( (p=1.49E-04) \),
ITGB5 (p=2.88E-06), M2BP (p=1.48E-06), MPO (p=1.97E-04), MMP3 (p=3.18E-02) and CRP (p=9.93E-07) are independently associated with PsA, while CD5L (p=8.71E-05), M2BP (p=9.38E-04) and MPO (p=3.27E-06) are independently associated with PsC.

Table 4.3 Polychotomous logistic regression analysis to identify biomarkers associated with PsA and PsC

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Homogeneity P-value(^a)</th>
<th>PsA OR (95% CI)</th>
<th>P-value(^b)</th>
<th>PsC OR (95% CI)</th>
<th>P-value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5L, ng/mL</td>
<td>5.48E-01</td>
<td>1.67 (1.28, 2.17)</td>
<td>1.49E-04</td>
<td>1.57 (1.25, 1.97)</td>
<td>8.71E-05</td>
</tr>
<tr>
<td>ITGB5, ng/mL</td>
<td>1.18E-05</td>
<td>3.23 (1.98, 5.27)</td>
<td>2.88E-06</td>
<td>1.21 (0.89, 1.64)</td>
<td>2.14E-01</td>
</tr>
<tr>
<td>M2BP, ng/mL</td>
<td>1.97E-03</td>
<td>151.73 (19.64, 1172.19)</td>
<td>1.48E-06</td>
<td>9.19 (2.47, 34.25)</td>
<td>9.38E-04</td>
</tr>
<tr>
<td>MMP3, ng/mL</td>
<td>1.72E-01</td>
<td>1.79 (1.05, 3.06)</td>
<td>3.18E-02</td>
<td>1.39 (0.86, 2.26)</td>
<td>1.81E-01</td>
</tr>
<tr>
<td>MPO, ng/mL</td>
<td>5.33E-01</td>
<td>2.36 (1.50, 3.72)</td>
<td>1.97E-04</td>
<td>2.64 (1.75, 3.98)</td>
<td>3.27E-06</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.40E-06</td>
<td>2.36 (1.67, 3.33)</td>
<td>9.93E-07</td>
<td>1.16 (0.91, 1.47)</td>
<td>2.28E-01</td>
</tr>
</tbody>
</table>

\(^a\)Indicates whether the markers have significantly different effects when modeling PsA and PsC separately, and controlling for age, sex, and the other biomarkers listed.

\(^b\)Indicates the significance of difference between PsA or PsC, and controls.

To further determine the significance of our putative PsA biomarkers, we compared the biomarker levels in patients with PsA, to those in patients with PsC, the results of which are demonstrated in Table 4.4. Consistent with the results previously described, ITGB5, M2BP, and CRP are independently associated with the presence of PsA when compared to PsC. ROC analysis of this combined model showed an AUC of 0.851 with a 95% CI of (0.799,0.904) (Figure 4.2).
Table 4.4 Logistic regression analysis comparing patients with PsA to PsC to identify biomarkers associated with PsA in patients with PsC

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>CD5L, ng/mL</td>
<td>1.08 (0.92, 1.26)</td>
<td>3.63E-01</td>
</tr>
<tr>
<td>ITGB5, ng/mL</td>
<td>4.07 (2.44, 6.81)</td>
<td>8.36E-08</td>
</tr>
<tr>
<td>M2BP, ng/mL</td>
<td>29.72 (5.88, 150.09)</td>
<td>4.05E-05</td>
</tr>
<tr>
<td>MMP3, ng/mL</td>
<td>1.59 (1.21, 2.11)</td>
<td>1.00E-03</td>
</tr>
<tr>
<td>MPO, ng/mL</td>
<td>1.09 (0.83, 1.43)</td>
<td>5.40E-01</td>
</tr>
<tr>
<td>CRP, mg/mL</td>
<td>1.93 (1.50, 2.48)</td>
<td>2.55E-07</td>
</tr>
</tbody>
</table>

Figure 4.2 ROC curve showing the AUC for the logistic regression model comparing PsA, to PsC patients
Additionally, after calculating Spearman’s rank correlation coefficients between the markers, we noted that although there are significant correlations between markers, their magnitudes are not large (Table 4.5). The largest correlation is between ITGB5 and M2BP (r= 0.24; p=3.51E-05).

**Table 4.5 Correlation between markers**

<table>
<thead>
<tr>
<th></th>
<th>CD5L</th>
<th>ITGB5</th>
<th>M2BP</th>
<th>MMP3</th>
<th>MPO</th>
<th>CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5L</td>
<td></td>
<td>0.15</td>
<td>0.04</td>
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*Indicates the Spearman’s rank correlation coefficient between markers*

*b Indicates the significance of correlation between markers; P<0.05 is considered significant*
4.4 Discussion

Psoriatic arthritis is an inflammatory arthritis that is associated with psoriasis, as it develops primarily in patients already diagnosed with chronic plaque psoriasis. PsA often leads to progressive joint damage and disability, poor quality of life, and increased mortality risk (32, 200), and early diagnosis and treatment may lead to better long term outcomes (79, 87-89). In the past, candidate biomarkers have been selected for validation based on their assumed importance in disease pathogenesis and the availability of assays (115), but to date, the need for discovery of PsA biomarkers in psoriasis patients, has remained unmet. To discover novel PsA biomarkers, we previously hypothesized that candidate markers of PsA are differentially expressed in inflamed skin and SF, and are detectable in serum. With this in mind, we performed high-throughput and targeted quantitative proteomics, as demonstrated in Chapters 2 and 3, to identify putative PsA biomarkers, and have verified a subset of those, in the current study. Our results indicate that ITGB5, M2BP, and CRP are associated with PsA and may serve as biomarkers for this disease.

Through our previously reported proteomic work, we identified increased ITGB5 levels in the inflamed skin of PsA patients, when compared to PsC. We now show that ITGB5 levels are elevated in PsA serum. ITGB5 has been described and investigated more extensively in synovial fibroblasts of patients with rheumatoid arthritis, where it is believed to serve as a ligand for Cyr61 (196). Cyr61 is a molecule secreted by fibroblast-like synoviocytes in the joint, and stimulates Interleukin-6 (IL-6) production via ITGA5-ITGB5/Akt/NF-κB signaling pathway (196). IL-6 production acts with IL-23 and TGB-β to trigger Th17 differentiation and IL-17 production (121, 122) in inflammatory processes, as they also occur in PsA. Here we show that
ITGB5 is a marker of PsA in PsC patients, where it may also play a role in the inflammatory nature of the disease.

M2BP (also known as galectin 3 binding protein) belongs to the family of scavenger receptor cysteine-rich domain proteins, and is a secreted glycoprotein suggested to have a role in host defense and tumour invasion (203-205). In addition to galectins, G3BP binds strongly with extracellular matrix components type IV–VI collagens, fibronectin, and nidogen, as well as with cell surface β1 integrins (206). M2BP has been investigated in the context of rheumatoid arthritis. Increased levels of M2BP were found in both synovial fluid and synovial tissue at sites of joint destruction, and M2BP levels correlated with the number of involved joints (207). It is believed that M2BP is a marker of synovial cell activation and joint destruction (207). However, M2BP was not elevated in the serum of patients with RA. We show that M2BP may also be involved in the pathogenesis of PsA, as it was elevated in both the synovial fluid (208), and serum of PsA patients, and serves as a specific marker of PsA in PsC patients.

While ITGB5 and M2BP are novel markers, CRP and MMP3 have previously been identified as markers of PsA in several pilot studies (11, 93, 115, 116). Here, we show that both CRP and MMP3 are increased in PsA patients, when compared to psoriasis, therefore supporting the previous data, that MMP3 and CRP are markers of PsA in patients with PsC.

Contrary to our previous findings outlined in Chapter 2, MPO and CD5L were not found to be elevated in patients with PsA, when compared to PsC. Similar results were obtained in a study by Ademowo et al. (209), whereby a panel of markers were identified and validated in synovial tissue biopsies, but when tested in serum, their findings could not be translated. The discrepancy in protein levels between tissues and serum could largely be due to the different analytical
techniques utilized to measure the markers. In targeted mass spectrometry, as discussed in Chapter 1, proteins are denatured, reduced, alkylated, and digested into peptides, some of which are then monitored. Unless specified, post translational modifications are not taken into account during multiple reaction monitoring assays. ELISAs are more sensitive in that respect. Due to the dependence on antibody-antigen interaction, a post translational modification of a protein may mask the epitope, thus limiting antigen-antibody binding, and lowering the signal.

Alternatively, since CD5L and MPO were both derived from the comparison of SF from PsA and early OA, our results may in fact reflect that, and could indicate that these markers may perform better in differentiating PsA from OA, and not PsA from PsC.

Thus, our results demonstrate that soluble biomarkers, previously identified through our proteomic studies, have the ability to distinguish PsA from PsC. Additionally, after examining the correlation between markers, we noted a significant, but low magnitude correlation between markers, which indicates that these biomarkers are independently associated with PsA. Moreover, logistic regression analyses provided evidence that these markers are independently associated with PsA. The diagnostic and prognostic roles of these biomarkers remain to be investigated in future longitudinal studies in patients with early PsA, and PsC alone.
Chapter 5
5 Summary and future directions

5.1 Summary of findings and implications

The preceding chapters of this thesis have described the necessity for identifying and developing biomarkers for psoriatic arthritis, in psoriasis patients. Prior to the work presented herein, candidate biomarkers have been selected for validation based on their assumed importance in PsA pathogenesis and the availability of assays, but to date, the discovery of clinically useful PsA biomarkers in psoriasis patients, has remained unmet. As such, in chapters two and three of this thesis we aimed to identify novel PsA candidate biomarkers, using quantitative mass-spectrometry-based proteomics. These represent the first studies that delineate the proteomes of synovial fluid and skin from patients with PsA, and led to the identification of 20 candidate PsA biomarkers. Seventeen of these markers were novel.

Following the discovery and verification of biomarkers, the candidate markers must undergo validation in serum samples. In chapter 4, I utilized enzyme-linked immunosorbent assays to measure the concentration of six of the proposed markers in a well-defined cohort of 300 individuals. I demonstrated that two novel markers, M2BP, ITGB5, and the well-known CRP are associated with PsA and may serve as biomarkers for this disease.

Therefore, through this work I was able to develop and utilize a standard biomarker discovery pipeline to identify novel biomarkers of psoriatic arthritis, in psoriasis patients. Additionally, and as important, these biomarkers may serve as interesting factors that could play important roles in the pathogenesis of PsA.
5.2 Limitations and considerations

Despite the interesting findings described, the project has several limitations. First, I utilized a label-free approach in order to quantify, and identify upregulated proteins in PsA target tissues. Potential disadvantages are lower reproducibility, which may compromise detection of smaller quantitative changes between samples. The lower reproducibility mostly results from the fractionation of peptides prior to LC-MS/MS analysis, and the subsequent pooling of eluted fractions, which can result in unequal/uncontrolled pooling. The reproducibility of LFQ experiments can be maximized by standardizing the entire pipeline, from sample collection and processing, to instrument setup and calibration, as I have done in the present study.

Second, pooling of samples in the discovery phase of this study could have potentially masked meaningful discrepancies among the compared proteomes. To minimize this, I confirmed all pool-derived using SRM in individual samples. By pooling biological samples as I have done, I obtained sufficient sample and increased the likelihood of identifying proteins that would otherwise be undetectable in individual samples, therefore allowing a more extensive proteomic coverage of the disease’s heterogeneity. Despite the shortcomings of the fractionation and pooling strategies, I do confirm the elevation of several markers using SRM assays, which provides validity to my entire strategy.

Third, the “control” synovial fluid described in Chapter 2, originated from joints of patients with early OA. Early OA synovial tissue has been shown to be inflammatory in nature, with increased mononuclear infiltration and overexpression of mediators of inflammation (210), so it does not represent the ideal comparison, as SF from PsC patients would. My use of early OA SF could have masked important inflammatory mediators otherwise not present in PsC SF, thus
interfering with the identifications of PsA markers in PsC patients. Additionally, as described, PsA is a heterogeneous disease with many clinical phenotypes, and by obtaining SF only from large joints, I narrowed my analysis to only one clinical phenotype, and excluded the distal interphalangeal predominant and spondylitis forms of PsA. Therefore, my findings may only be applicable to the asymmetric or symmetric forms of PsA.

Fourth, to ensure I chose protein markers that were specific to the pathogenesis of PsA, I filtered my proteins, as described in Chapter 2 and 3. I narrowed my results to include only upregulated proteins, proteins that are not present in high abundance in the serum, and proteins expressed or present in cells/tissues associated with PsA. This represents another limitation, since by doing so, I may have disregarded potentially important proteins of interest. For example, SAA (which was excluded due to its high abundance in serum in Chapter 2), has been shown to have important biological effects in driving inflammation in rheumatoid arthritis (211, 212).

Finally, the three biomarkers that were validated only included one of the previously identified PsA biomarkers by Chandran et al. (115), which may be due to the different and much larger validation cohort that was utilized in the current study. This fact highlights the importance of multiple and multi-site validation cohorts, in determining the clinical utility of the possible PsA biomarkers that have been named in this study and others.

Despite the limitations, the project carries several strengths. The first lies in the quality and source of the samples analyzed. Any biomarker discovery pipeline relies on the use of well-characterized, high-quality samples. It is ideal to collect and store samples following a standardized protocol, which is extremely important during the biomarker development process considering that a candidate biomarker needs to be repeatedly verified/validated with large and
independent sample sets. The synovial fluid, skin, and serum samples used in the current studies were obtained from one institution and underwent strict sample handling procedures.

Second, the discovery phase of our biomarker development pipeline, was followed by SRM-based verification of a large number of candidates, which was followed by ELISA-based validation of three markers in serum samples from a well-defined cohort of 300 patients. The work presented in this thesis represents the most comprehensive biomarker development project conducted to date for PsA.
5.3 Future directions

In this thesis, 20 proteins were presented that may represent PsA biomarkers, and only 7 of those were tested in serum, largely due to the lack of commercially available antibodies or immunoassay kits. Apart from CRP, S100A9, and MMP3, none of the markers proposed have been previously measured in serum from PsA patients. Therefore, an important next step would be to develop monoclonal and polyclonal antibodies to allow the measurement of the remaining candidates in PsA, PsC, and healthy serum.

Protein verification/validation was only performed on upregulated proteins, proteins specific to PsA cells/tissues, and proteins not present in high abundance in the serum. The proteins I excluded are also of importance, as they may represent mediators/biomarkers of PsA, and in future studies, these should be further investigated to delineate their involvement (or lack of involvement) in PsA.

As described, the validation cohort consisted of patients with PsA, PsC, as well as healthy controls. Since the ultimate scope is to identify markers of early PsA in psoriasis patients, future longitudinal studies consisting of patients with early PsA, and psoriasis alone are warranted to evaluate the diagnostic and prognostic roles of these biomarkers.

Since PsA is a largely heterogeneous disease, it is unlikely that a single biomarker will be sufficient in predicting PsA in psoriasis patients. There are a number of markers, as exemplified earlier, that have been tested in pilot studies, and are still awaiting validation in large patient cohorts. Therefore, another important step would be to evaluate these, alongside those markers discovered and described in this thesis, in order to compose panels which are likely more
informative and reliable. Also, there are some clinical features in psoriasis that indicate higher risk for developing PsA, such as nail psoriasis, scalp, intergluteal/perianal psoriasis, and severe psoriasis. Combining these clinical features, with biomarker panels may represent the ideal predictive model to identify psoriatic arthritis.

Lastly, the work presented in this thesis demonstrated two newly-identified PsA-associated markers: ITGB5, and M2BP. Studies examining the mechanism resulting in the elevation of these proteins will be important, as they may provide novel insights into PsA pathogenesis.
5.4 Conclusion

Early diagnosis and management of PsA is known to lead to better long-term patient outcomes (79, 87-89). Unfortunately identifying inflammatory musculoskeletal disease early in patients with psoriasis is a very difficult task, often due to a lack of validated biomarkers. Therefore, much of the recent research in PsA, has focused on identifying, serum-based biomarkers, which could be used to screen psoriasis patients, in order to identify those that may have or will develop PsA. The work described in this thesis has outlined a mass-spectrometry-based approach to PsA biomarker discovery, and has resulted in the validation of two novel biomarkers, adding to the growing list of PsA markers. Large multi-site validation cohorts are imperative in finally identifying clinically relevant PsA biomarkers.
References


165. Martínez-Morillo E, Cho CK, Drabovich AP, Shaw JL, Soosaipillai A, Diamandis EP. Development of a multiplex selected reaction monitoring assay for quantification of


205. Krautbauer S, Eisinger K, Hader Y, Buechler C. Free fatty acids and IL-6 induce adipocyte galectin-3 which is increased in white and brown adipose tissues of obese mice. *Cytokine* 2014;69:263-71.


