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Inflammatory Profiling in Early Osteoarthritis

by

Guomin Ren

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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Abstract

Osteoarthritis (OA) is one of the most common chronic diseases worldwide which can lead to disability. There is a desperate need for the efficient and reliable detection of OA at the early stage when patients are likely to benefit most from disease interventions. It has been shown in previous studies that inflammation plays important roles in cartilage degeneration, synovitis, remodeling of the subchondral bone and pain. The purpose of this thesis was to determine if a panel of inflammatory cytokines were distinct within individuals with pre-radiographic OA and/or an increased risk of developing OA.

Serum inflammatory profiles were analyzed within a number of patient cohorts [i.e., radiographic OA patients (hip and knee), youth with a history of intra-articular knee injury, corresponding controls]; and it was found that inflammatory profiles were distinct between knee vs. hip OA patients. Additionally, a computation method was developed which identified a coordinated change in cytokine profiles in the youth knee injury cohort. This computational methodology highlighted a number of candidate biomarkers that contributed to this observed difference, including C-C motif chemokine 22 (CCL22)/macrophage derived chemokine (MDC) which was selected for further study. In a pre-clinical rat OA model, it was found that CCL22 plays a functional role in chondrocyte apoptosis and cartilage degeneration. Further, it was found that CCL22 treated synovial fibroblasts demonstrated altered expression of inflammatory factors.

These results suggested that CCL22 may be a biomarker and potential drug target in early OA. These results also suggested that CCL22 may be associated with OA pain, yet this was not examined directly and an *in vivo* model where CCL22 expression could be regulated would be required to test this hypothesis. While it was observed that CCL22 is expressed in damaged cartilage and acts on human chondrocytes and synovial fibroblasts, additional studies are required to determine how CCL22

triggered these changes in synovial fibroblasts as these results suggest this is CCR4 independent. Furthermore, it would be essential to validate these findings in an independent cohort to examine the sensitivity and/or specificity of CCL22 as an early OA biomarker.

Preface

Chapters 2-6 are based on scientific manuscripts which are either in preparation, submitted or published.

Chapter 2 is published in *Biomarkers*: Ren G, Krawetz R. Applying computation biology and "big data" to develop multiplex diagnostics for complex chronic diseases such as osteoarthritis. Biomarkers. 2015;20(8):533-9.

Chapter 3 is published in *BMC Musculoskeletal Disorders*: Ren G, Lutz I, Railton P, Wiley JP, McAllister J, Powell J, Krawetz R. Serum and synovial fluid cytokine profiling in hip osteoarthritis: distinct from knee osteoarthritis and correlated with pain. BMC Musculoskelet Disord. 2018;19(1):39.

Chapter 4 has been submitted to *Molecular Diagnosis & Therapy*: Ren G, Krawetz R. Biochemical markers for the early identification of Osteoarthritis: systematic review and meta-analysis. MDTA-D-18-00135, revision requested.

Chapter 5 has been submitted to *Cytokine*: Ren G, Whittaker JL, Leonard C, De Rantere D, Pang D, Salo P, Fritzler M, Kapoor M, de Koning J, Jaremko J, Emery C, Krawetz R. CCL22 is a biomarker of cartilage injury and plays a functional role in chondrocyte apoptosis. CYTO-18-556.

Chapter 6 is in preparation for submission: Ren G, Al-Jezani N, Railton P, Powell J, Krawetz R. CCL22 induces inflammatory changes in synovial fibroblasts from healthy donors but patients with Osteoarthritis.

Author's Contribution

Chapter 2:

Literature review, data analysis and interpretation, drafting the manuscript.

Chapter 3:

Study design, sample preparation, data collection and statistical analysis, histology and immunofluorescence, drafting the manuscript.

Chapter 4:

Literature review, data analysis and interpretation, drafting the manuscript.

Chapter 5:

Study design (all aspects except PrE-OA cohort), statistical model development, data collection and statistical analysis, blood sample preparation and Luminex analysis (rat), histology and immunofluorescence, drafting the manuscript.

Chapter 6:

Study design, primary cell culture, flow cytometry, data collection and statistical analysis, drafting the manuscript.

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At last I wish to thank all the funding agencies that provided scholarship support for my research including the Arthritis Society, University of Calgary Graduate Studies and the Biomedical Engineering Graduate Program.

Dedication

I would like to dedicate this thesis to my family.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ACL	Anterior cruciate ligament
ACR	American College of Rheumatology
BMI	Body Mass Index
CI	confidence interval
COMP	Cartilage oligomeric matrix protein
CTX-II	Collagen type II
DMM	Destabilization of the medial meniscus
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	The enzyme-linked immunosorbent assay
ES	Effect size
FAI	Femoroacetabular impingement
FGF2	Fibroblast growth factor 2
GRO	Growth-regulated protein
GWAS	Genome-wide association study
HA	Hyaluronic acid
HHS	Harris Hip score
HOOS	Hip disability and osteoarthritis outcome score
ICOAP	The intermittent and constant pain score
IKK	IκB kinase

IL	Interleukin
IP	Interferon gamma-induced protein
JNK	c-Jun N-terminal kinase
K/L	Kellgren and Lawerence
MCP	Monocyte chemoattractant protein
MDC	Macrophage-derived chemokine
MIP	Macrophage inflammatory protein
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MWW	Mann–Whitney–Wilcoxon test
NLR	NOD-like receptors
OA	Osteoarthritis
PCA	Principle component analysis
PIIANP	N-terminal pro-peptide of collagen IIA
PPI	Protein-protein interaction
PrE-OA	The Alberta "Prevention of Osteoarthritis Following Joint Injury in Youth Sport" cohort
PRR	Pattern-recognition receptors
PTOA	Post-traumatic osteoarthritis
RA	Rheumatoid arthritis
ROC	Receiver operating characteristic
SF36	Short Form 36

SF	Synovial fluid
SMD	Standardized mean difference
Th	T Helper
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TRADD	TNFRSF1A Associated Via Death Domain
UCLA	University of California Los Angeles activity score
VEGF	Vascular endothelial growth factor
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index

Chapter One: Introduction

1.1 Background

Osteoarthritis (OA) is a chronic disease which is characterized by the progressive degeneration of the articular cartilage. It is the leading cause of mobility disability in people aged 65 years and older worldwide (1). In Canada, it affects approximately 12% of the population. Within Alberta alone, productivity loss related to OA had a negative economic impact of $2.75 billion in 2010 (2). It is estimated that by 2040, more than 1 in 4 Canadians will have OA, thereby affecting 30% of the labor force (2). While OA typically develops over years to decades, current clinical diagnosis of OA, which relies heavily on symptomatic (pain, loss of motion) and radiographic assessment (cartilage loss, joint space narrowing and osteophytes) can only be reliably made at a late/advanced stage of the disease (3). The main limitation of clinically diagnosing OA at later stages is at that point in the disease pathology, disease-modifying options are limited (4). Just how little we truly understand about the onset and pathogenesis within the early stages of OA, is demonstrated by the fact that over the last 40+ years of research into OA, no single diagnostic test for early (pre-radiographic) OA has been approved by the FDA or Health Canada as none have been demonstrated to be specific or sensitive enough to comprehensively classify early-stage OA patients (5).

1.2 Risk factors of OA

Identifying individuals at a high risk of developing OA is critical to provide early

disease identification/intervention aiming at reducing socioeconomic impact of OA by precisely allocating limited health-care and research resources. The multifactorial pathogenesis of OA, including aging, injury, and genetic predisposition may all act as contributing factors to the onset/progression of OA. Broadly, obesity is also widely accepted to have a strong association with the risk of developing OA (6). It should also be noted that many patients with OA would present with many of these and/or other risk factors making it difficult to tease out exactly how each one or combination(s) are increasing the risk of developing OA; or potentially an increased risked of accelerated progression of OA. However, risk factors such as injury and abnormal joint morphology are better understood. Previous studies report that 12-20 years post knee injury, there is a 10-fold increased risk of developing knee radiographic OA, compared to an uninjured population (7). In terms of abnormal morphology, infants with developmental dysplasia of the hip will have an increased chance to develop hip OA in adulthood (8), while femoroacetabular impingement has been also demonstrated to lead to cartilage wear and the eventual onset OA (9). These examples and many others (not covered in this thesis), demonstrate that onset and progression of OA is driven by numerous factors, often in combination with one another, and this has made the early identification of patients with OA a challenge to both health care providers and researchers. As previously stated, there are many potential risk factors for developing OA, however, in this thesis, more background will be provided for the two main factors (injury and abnormal joint morphology) central to the hip and knee cohorts being employed.

1.2.1 Joint injury

While idiopathic OA typically develops in older adults, post-traumatic osteoarthritis (PTOA) has been shown to develop in young to middle-aged adults 12-20 years after joint injuries sustained in youth (10). Prospective studies report that knee injury increases the risk of developing radiographic knee OA 10-fold (11). It is estimated that 12% of cases of symptomatic OA in the hip, knee, and ankle are due to previous injury. Moreover, evidences have shown that more than 50% of individuals with an anterior cruciate ligament (ACL) tear or meniscus injury go on to develop knee OA (12, 13). At present, there is a paucity of research examining outcomes early (<10 years post-injury) in the period between joint injury and disease onset when interventions have potential to delay or prevent progression to OA (4). Therefore, once joint injury has occurred it is imperative that individuals who have yet to display clinical signs of OA, but who are at high risk of developing the disease, are included in research studies as they may hold vital clues understanding the early/pre-structural changes in the disease. Understanding these processes may aid in the detection of early OA and development of interventions aimed at slowing disease progression.

1.2.2 Bone abnormalities:

Research over the past decade has revealed that some cases of idiopathic OA may be due to abnormal hip anatomy/morphology, such as dysplasia and femoracetabular impingement (14). When bones within the joint are abnormally shaped, they do not fit together properly, and therefore can cause damage to the joint. Hip dysplasia is a

developmental deformation of the femoral head, acetabulum, or both, caused by a developmental disorder in which the hip joint forms incorrectly during childhood. Several previous prospective studies have shown acetabular dysplasia is significantly associated with an increased risk of developing hip OA (15).

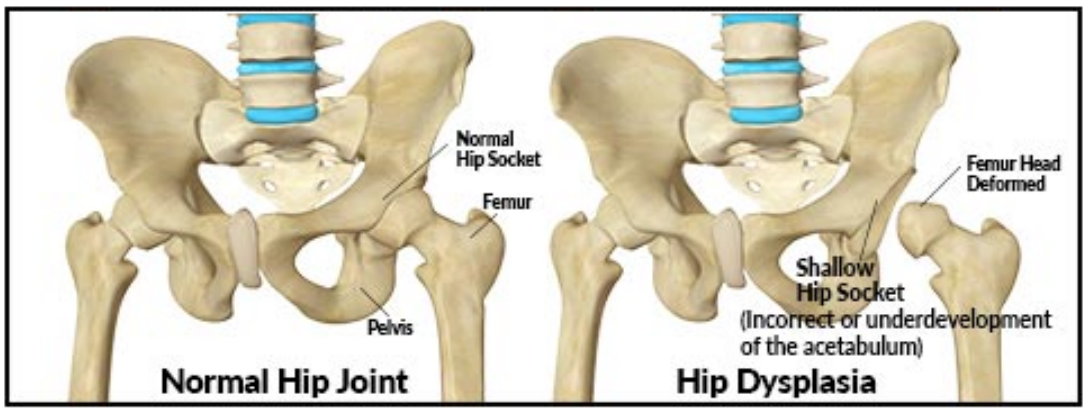


Figure 1-1. Normal hip (left) and dysplasia hip (right) (adapted from (16)).

Femoroacetabular impingement (FAI) is another common bony abnormality which is associated with OA. In FAI joints, the abnormality leads to atypical stresses between labrum and cartilage, these stresses separate the labrum from the cartilage as the labrum is pushed outwards and the cartilage is pushed centrally, this process eventually leads to articular degeneration and finally OA of the hip (14, 17). One subtype of FAI - Cam impingement (a nonspherical, cam-shaped abnormality which is caused by the extra bone formation at the anterolateral head–neck junction) has been strongly associated with the development of hip OA (18).

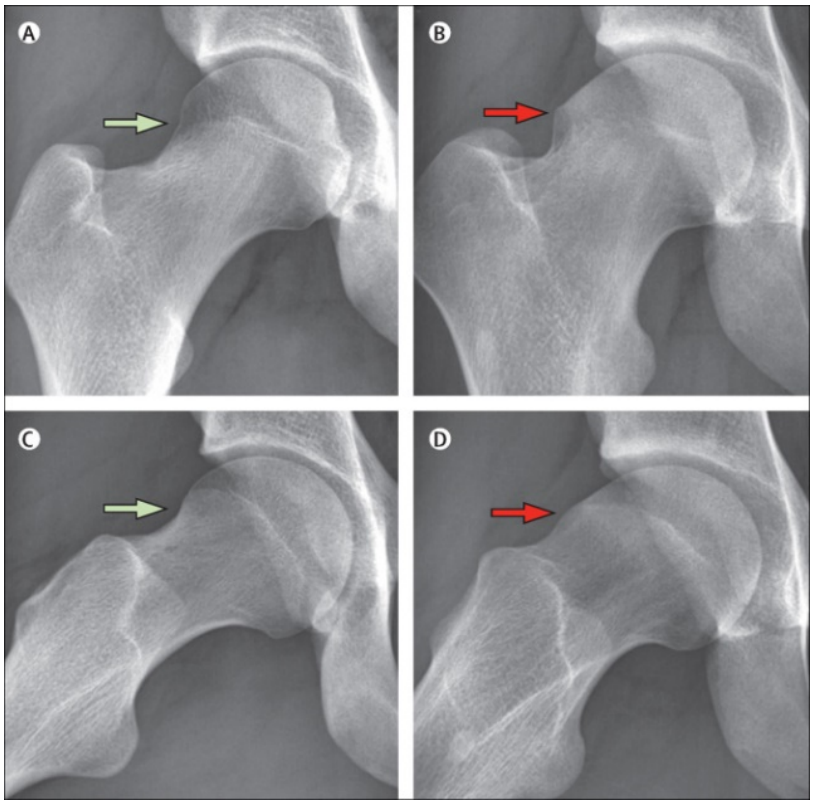


Figure 1-2. Normal hip (A and C) and cam lesion femoroacetabular impingement morphology (B and D) (from (19)).

While other risk factors, such as obesity/metabolic disorders, heart disease, depression, smoking, and other co-morbidities, can't be discounted to play a significant role in onset/development of OA with/without prior injury and/or abnormal bone morphology (and therefore contribute to the observations in this thesis); it is not feasible and in some cases not possible to obtain and/or control for the complete/accurate heath status (or history) in a given patient. However, a number of questionnaires were employed in when the cohorts were developed that are employed in this study to ascertain as much clinical data as possible on these individuals.

1.3 OA is an inflammatory disease

Unlike other common forms of arthritis such as rheumatoid arthritis and psoriatic arthritis, which are generally categorized as autoimmune diseases, OA has long been considered as a mechanical “wear and tear” disease. However, recent studies have shown that physical wear alone is not necessarily the main driving force in the onset and development of OA and that inflammation plays a critical role (20). In the mouse model, Lapveteläinen et al. have found that the heavy use of the joint (lifelong voluntary wheel running) does not accelerate OA; on the contrary, it appears to protect the joint from the OA (21). Human studies have also observed similar results (22). Furthermore, after anterior cruciate ligament (ACL) tear, ACL reconstruction surgeries are often recommended to maintain patient’s knee joint stability by preventing abnormal mechanical loading on the cartilage; yet, it is clear that ACL reconstruction does not stop OA, even though it restores the stability of the joint to some extent (23).

These kinds of observations have led the research community to ask: If it is not physical wear, what are the main contributors to the development of OA? One of the most accepted hypotheses is that OA starts from physical stimuli/trauma (e.g. knee injuries, overload of the joints, bone abnormalities). These stimuli can trigger complex inflammatory responses, which in turn disrupt the biochemical homeostasis of articular cartilage and other joint tissues, perpetuating the degeneration of the articular cartilage and structural/functional changes throughout the whole joint (20).

1.4 Initiation of the inflammatory response in OA

Multiple pathways are involved in the initiation of the inflammatory response OA in both humans and pre-clinical models. The innate immune system, also known as non-specific immune system, comprises the cells and mechanisms that defend the host from infection by foreign agents/organisms (24). This system can also trigger a sterile inflammation response without an exogenous pathogen. This is achieved by the binding of pattern-recognition receptors (PRRs) and damage-associated molecular patterns (DAMPs, also known as alarmins), released by stressed cells/damaged tissues as endogenous danger signals (e.g. chondrocytes undergoing necrosis following acute joint injury) (25). PRRs include membrane-bound PRRs such as Toll-like receptors (TLR), cytoplasmic PRRs such as NOD-like receptors (NLR), and secreted PRRs such as complement receptors (26). DAMPs vary greatly depending on the injured tissue and the type of cell. They can be remnants of cellular breakdown (e.g. DNA fragments) or extracellular matrix breakdown products (e.g., hyaluronan, collagen). Activation of PRRs triggers cell signaling that leads to the production of inflammatory cytokines, and the induction of downstream inflammatory responses. Other than PPR-mediated inflammatory pathway, the complement system, which is made up of a large number of distinct plasma proteins that react with one another to opsonize pathogens, can also induce a series of inflammatory responses that help to fight infection (27). The present of abundant pro-inflammatory cytokines in areas of OA damaged, further lead to macrophage and CD4+ T cell infiltrate into inflamed joint tissue (20). In the synovium of OA patients, it has been found that CD4+ Th1 subtype and macrophages are most

abundant cell types. These cells are responsible for the enhanced stimulation of innate immune cells and the activation of B cells which increase/perpetuate inflammation by producing autoantibodies specific for cartilage cell surface proteins such as osteopontin and collagen (28).

1.5 Biochemical biomarkers in OA:

Over the last 40+ years of research, no single biomarker for early (pre-radiographic) OA has been approved by the FDA or health Canada as none have been demonstrated to be specific or sensitive enough to comprehensively classify early-stage OA patients (5). Biochemical biomarkers are an ideal candidate for asymptomatic early stage OA as they may directly or indirectly participate in the biochemical processes of onset and/or progression of the disease. Therefore, it may be possible that inflammatory biomarkers which are likely upstream of cartilage breakdown products may be more accurate than traditional clinical measurements such as cartilage thickness or pain that would be downstream of the inflammatory cascade. In recent years, many studies have been conducted focusing on various OA biochemical biomarkers and a subset has been summarized based on studies that presented accuracy of their biomarkers in **Table 2-1** and **Table 4-1**.

1.5.1 Metabolites as biomarkers:

One common type/class of biomarkers that have been investigated is joint tissue metabolites. It has been observed that these metabolites are released into bodily fluids

(serum/plasma/synovial fluid) during tissue turnover (29) and therefore their concentration may directly reflect the metabolic rate of the joint tissues affect by OA.

Cartilage oligomeric matrix protein (COMP) is non-collagenous extracellular matrix (ECM) glycoprotein which contains thrombospondin-like domains that can bind to different collagen types (30) and is responsible for collagen–collagen interactions and microfibril formation in the cartilage. It is believed that COMP is mainly produced by articular chondrocytes and its elevation in body fluid is related to cartilage damage. Serum COMP concentration has been found to be elevated in a number of independent OA cohorts, and has correlated with the severity of OA (30-33). Recent evidence has demonstrated that an elevated concentration of COMP can be detected in the synovial fluid of injured joints years after the injury (34) or in the serum of OA patients after physical exercise (35). These results and others suggest that COMP may be sensitive biomarker and might have potential for detecting the asymptomatic, early stages of OA. Further studies are underway to verify these results in the same youth injury cohort (PrE-OA) employed in this thesis.

C-terminal telopeptide of collagen type II (CTX-II) is another commonly investigated OA biomarker. CTX-II is derived from the C-telopeptide portion of collagen type II. Since collagen type II is a major structural component of articular cartilage (60% of dry weight of cartilage) and cartilage is highly enriched for collagen type II expression vs. all other tissues (36), the fragments of collagen type II such as CTX-II might be representative biomarkers for cartilage degeneration/turnover and potentially for early OA(37). Similar to COMP, an increase of CTX-II has been observed in many

independent OA cohorts (38-41). Other type II collagen degradation products including collagen type II cleavage (C2C), matrix metalloproteinase-derived fragments of type II collagen (C2M), Coll2-1, Coll2-1-NO2 have been investigated in many studies, however, as diagnostic biomarkers for early OA, the reliability and accuracy vary among studies (42, 43).

While most previous studies have focused on cartilage degeneration biomarkers, there are a few studies that have focused on cartilage synthesis biomarkers. Chondroitin sulfate epitope 846 (CS846) is an epitope present in newly synthetized aggrecan molecules. It is observed to be increased in OA patients (44) and after joint injury (45). These studies indicate that the synthesis/turnover of the proteoglycan content within the cartilage is increased in OA. N-terminal pro-peptide of collagen IIA (PIIANP), spliced from type II pro-collagen, is a biomarker for a collagen type II synthesis (46). Serum PIIANP is found decreased in patients with a greater burden of hip and knee OA (47), suggesting a decreased collagen synthesis in OA which is in opposition to the studies on CS846/proteoglycans. However, it should be noted that the changes of these biomarkers do not necessarily reflect a pathological increase or decrease in turnover of newly formed cartilage matrix. In fact, by using a radiocarbon dating method, Heinemeier et al. have shown that cartilage (specifically the collagen matrix) does not turnover after skeletal maturity regardless the occurrence of OA (48).

1.5.2 Inflammatory Markers:

While many of the commonly studied biomarkers in OA are based on the turnover of

the cartilage itself, an increased number of inflammatory markers have come to attention recently as the role of inflammation in OA onset/progression has become clearer. The inflammatory response can be triggered by the debris of damaged tissues within the joint (e.g. cartilage) as discussed earlier. Therefore, all the molecules (DAMPs, PPRs, cytokines and etc.) within these signaling pathways could potentially have use as biomarkers for detecting inflammation which may in turn lead to the onset and/or progression of OA. If it can be determined that a given inflammatory pathway is specific and or sensitive to OA vs. other diseases and/or general injury, then these may indeed have diagnostic potential in early OA. In the following paragraphs, a number of potential biomarkers native to the synovial joint will be introduced and how these factors may play a role in regulating the inflammatory response in OA will be discussed.

Hyaluronic acid (HA), also known as hyaluronan, is a major component of synovial fluid and the extracellular matrix of the cartilage. It is believed that large molecular weight HA is responsible for lubricating the joint. However, in post-trauma or OA joints, the average molecular weight of HA and the viscosity of synovial fluid decreases significantly compared to normal individuals (49). Furthermore, small molecular weight (and potentially) HA fragments stimulates inflammation by binding to a number of cell surface PPRs (such as TLR4 and TLR2 as well as CD44) on various cell types including both leukocytes and non-leukocytes (such as fibroblasts and mesenchymal stem cells). While the molecular weight of HA decreases in OA, the overall abundance of HA was found to be elevated in multiple OA cohorts (50-53).

Proteoglycan 4 (PRG4/lubricin) is secreted by cells that reside in the superficial layer of articular cartilage and also within the synovium. It is generally accepted that lubricin has a protective effect on the cartilage due to its lubricating abilities. However, recent studies have demonstrated that lubricin may play a role in modulating inflammatory responses through interaction with CD44 and TLRs and regulate pro-inflammatory pathways in an HA independent manner (54).

S100A8, S100A9, and S100A12 are a subgroup of the S100 protein family which are intracellular calcium-binding proteins that control key cellular pathways including the inflammatory response. In an antigen-induced arthritis mice model, the lack of S100A8/A9 prevented cartilage degradation, indicating that these proteins play a major role in cartilage degradation and development of inflammatory arthritis. Human studies suggest that chondrocytes respond to extracellular S100A8/S100A9 stimulation by upregulating catabolic markers (MMPs, IL-6, IL-8) and downregulating anabolic markers (aggrecan and type II collagen) through TLR-4-independent pathways (55). An *in vitro* study demonstrated that S100A12 treatment can promote MMP-13 and VEGF expression in human OA chondrocytes through a p38 MAPK and NF-κB dependent pathway (56). S100A12 is also found present in synovial fluid in knee of OA patients, and correlated with WOMAC scores, indicating that S100A12 might be a potential biomarker of OA severity. Zreiqat et al. demonstrated that S100A8 and S100A9 expression is upregulated in early OA but reduced in late OA, suggesting that these proteins may initiate cartilage degradation by upregulating MMPs and ADAMTSs and hence the expression and/or activity of s100 proteins may also have potential as early

OA biomarkers (57).

Cytokines are almost universally recognized as indicators of inflammation (pro and/or anti) as they are the major inflammatory signal mediators (58). Therefore, they deserve significant study to determine if any (or combination of any) cytokines may be potential biomarkers for OA. After the activation of innate immune signaling pathways through PRRs, a variety of cell types in joint tissues including (but not exclusively) synovial fibroblasts, macrophages, and chondrocytes start to produce soluble inflammatory mediators including both pro-inflammatory (e.g. IL-1β, TNFα, IL-6, IL-15) and anti-inflammatory (e.g. IL-6, IL10, IL-13) cytokines (59-61). IL-1β and TNFα are considered key cytokines involved in the pathogenesis of OA. They are secreted by chondrocytes, osteoblasts, synoviocytes (type A and B cells) and mononuclear cells which infiltrate into joint during the inflammatory response. In patients with OA, levels of both IL-1α and TNFα are elevated in the synovial fluid, synovial membrane, subchondral bone and cartilage (22). The receptors of Il-1β include IL-1R1 and IL-1R2, with IL-1R2 becoming inactive after binding to IL-1β. IL-1R1 belongs to TLR family, with binding of IL-1β with IL-1R1 triggering a MyD88 dependent pathway similar to TLR4 (62). This signaling cascade ends with the activation of the transcription factors NF-κB, p38MAPK and c-Jun N-terminal kinase (JNK) (62). The expression of the IL-1R1 is increased in patients with OA on the surface of chondrocytes and fibroblast-like synoviocytes (FLS) compared to treatment groups (23). TNFα binds to two receptors on the cell membrane, TNF receptor I (TNFRI or p55) and TNFRII (or p75). The binding of TNFα and TNF-R1 causes interaction between the TRADD adapter protein

with the DD domain and gradual binding of other adapter proteins such as TRAF2 and RIP, which then activate the multicomponent protein kinase IKK, resulting in the phosphorylation of the IKK complex and ultimately, the activation of the NF-κB pathway. However, because of the complexity (e.g. redundancy, routes of feedback and cross-talk) within cytokine signaling networks, it is difficult to understand the role of a given cytokine when studied in isolation and therefore, it might more comprehensive/informative to study multiple cytokines at the same time. In a previous study, by comparing the inflammatory profiles (38 cytokines), Heard et al., successfully classified OA and normal patients at considerable high sensitivity and specificity which is impossible to achieve with the use of any of these individual cytokines alone (63).

As stated earlier, current clinical diagnosis of OA relies heavily on physical examination (e.g., pain, joint function/stiffness) and radiographic assessment (e.g., joint space narrowing) (64, 65). However, these symptomatic and structural changes can only be observed after degeneration of the joint tissue has already progressed and may be irreversible (3). Therefore, one of the most important characteristics of a novel OA (diagnostic) biomarker(s) is the ability to detect the early (pre-radiographic) phase of OA. Unfortunately, OA patient cohorts included in most of these studies discussed in this chapter, were identified based on post-radiographic OA criteria (K/L grade >= 2). One main reason for studies selecting advanced OA patients is that it is inherently difficult to detect pre-radiographic OA (hence a vicious circle), the approach to address this gap in the literature, was to employ a longitudinal study on a high risk group (joint injury) specifically the PrE-OA study group (**https://ucalgary.ca/siprc/files/siprc/pre-**

oa.pdf) (66). The PrE-OA group consists of 100 youth adults who had sustained a sport-related intra-articular knee injury 3–10 years previously and 100 uninjured age, sex and sport (at time of injury) matched controls. Previous analyses suggested participants with history of injury suffered from more symptoms and poorer function, and are at greater risk of being overweight/obese 3-10 years post-injury compared to matched uninjured controls (66-68). Utilizing this type of cohort is essential to examine potential early OA biomarkers in patients when they have not developed radiographic OA and then clinically classify them when some of them develop radiographic OA years later. Therefore, longitudinal studies looking at the association between biomarkers and pre-radiographic indicators such as MRI OA score and joint pain without radiographic symptoms are essential for developing new early OA biomarkers.

1.6 High-throughput Technology in OA diagnostics:

The complexity of immune activation and inflammation in the pathogenesis of complex diseases is becoming increasingly well recognized. Traditional studies focusing on quantitation of only one or a few biomarkers per study in correlation with disease state has become far from efficient. High-throughput techniques have demonstrated significant potential for biomarker development in diseases such as cancer (69, 70), cardiovascular disease (71, 72), Alzheimer's (73) and other chronic diseases (74, 75). The most popular high-throughput technologies include RNA-seq (transcript identification), Mass spectrometry (MS) (protein identification), and multiplex bead

arrays (protein identification). RNA-seq provides a revolutionary way to study transcriptome during the onset of diseases. It can also unveil some information at DNA level such as single nucleotide variants (76). Due to high cost and the low representativeness at the protein level, RNA-seq hasn't been applied as widely as other technologies. MS based high-throughput technologies aid with the identification of differentially expressed proteins and their post-translational modifications during disease progression which can then be further examined/validated as biomarkers for early diagnosis and monitoring disease treatment (77). Despite the advances in the biomarker discovery application of MS, limitations still remain: MS demonstrates relatively low sensitivity (>ug/ml) in detecting serum analytes (usually range between 50 pg/mL ~ 10 ng/mL) (78). Therefore, a number of studies interested in protein identification and quantification employ high-throughput ELISA technology (multiplex assays) such as Luminex and Meso Scale to examine panels of proteins (typically numbering between 20 and 60 an analytes per assay, with a current hardware limit of 500 protein examined in a single assay) (79). Multiplex array technology is more sensitive (~pg/mL) than MS and therefore allows detection of minor perturbations of analytes which haven't been recognized previously by other methods.

High-throughput technologies have also demonstrated promise for the development of OA diagnostics at the advanced/late stage by our group and others using a broad range investigation of inflammatory profiles in different tissues and body fluids (80). Some of these studies successfully classified OA and normal patients at considerable high sensitivity and specificity by comparing their inflammatory profiles (e.g. not relying on

single analytes/biomarkers) (63). Serum and urine are the most commonly used body fluids for proteomic and metabolomics analysis of OA and have the most clinical potential as the access to them are non-invasive (81). However, since they are spatially removed from the affected tissues it is possible that some key proteins may be diluted. Synovial fluid (SF), although difficult to obtain, can be studied as a compromise of non-invasiveness and sensitivity (82).

The expansion of high-throughput technology application has brought its own challenges to biomarker discovery; analysis of a huge quantity of data (includes not only values but also relationships between values and biological meaning behind them). In the language of classical statistics, the traditional discovery of biomarkers from high-throughput data can be modeled as selecting the most discriminating features (or variables) for classification and then testing whether the multivariate set of selected biomarkers differs between disease and control populations. In the last few years, high-throughput studies of biological systems are rapidly accumulating a wealth of 'big-data', including single-interactome (e.g. genomics, transcriptomics, proteomics, metabolomics), interactions between single-interactome (e.g. protein-protein interaction) and interaction cross interactomes (e.g. signal pathway). The integration of data from different scales would greatly enhance the accuracy and efficiency of the analyses. For example, in proteomic data analysis, data dimensions could be reduced by removing the redundant proteins if it can be demonstrated that they have similar functions in the same signaling pathway. Another example is that some proteins have a non-linear relationship as they need to bind together to form a protein complex in order

to be functional, lacking any of them will lead to the interruption of the signal pathway. However, to integrate these complex relationships between variables is beyond a classical statistical approach, and therefore a new field of study “systems-biology” has emerged to fill the analyses gap created by “big-data”.

Systems biology is an inter-disciplinary field of study that focuses on complex relationships within biological systems or biological experiments using computational modeling. The powerful capability of data analysis and arrangement makes systems biology an ideal tool for biological high-throughput “big-data” analysis. Network-based approaches have recently emerged as one of the powerful systems biology tools for biomarker discovery studies and understanding complex biological systems (83-85). One such study combined time-course microarray data and prior knowledge (protein-protein interaction network), to identify dynamical network biomarkers (a group of proteins) for influenza, acute lung disease, and type 2 diabetes. These markers served as an early warning signal during disease initiation and progression with high sensitivity and accuracy (86). Current network-based approaches have limitations: most of them view networks as being fully connected without considering the actual network connections or merely downloaded biomolecular interaction networks from various databases (e.g. two proteins resident in different tissues will never interact *in vivo*, but still may be identified as binding partners *in silico*). Moreover, in living biological systems, even within one pathway, signals transfer back and forth between pathways comprising the entire cellular environment and these networks can change over time, which cannot be easily accounted for in a model. Current network models which are

constrained in single-interactome are limited in the analysis of entire disease progression. In a novel work conducted by Karagoz *et al*., the authors proposed a method combining transcriptomic and proteomic data to discover transcriptional control mechanisms of triple negative breast cancer (87). Overall, by taking advantage of massive computational resources and advanced data processing and analyses methods, system biology approaches to diagnostic medicine and biomarker development has had successes in the early detection of complex chronic diseases and in some cases has given us a deeper understanding of the molecular pathogenesis of disease (88).

1.7 Limitation of Current OA diagnostic studies:

Even though extensive high-throughput data has been developed to date in OA research, and many potential biomarkers have been proposed in the literature; their sensitivity, specificity and/or reliability has not reached a level sufficient for clinical use (89, 90). One possible explanation for this deficiency of biomarkers is the multifactorial pathogenesis of OA. Age, injury, genetic predisposition and additional factors (not covered) all may act as contributing factors in the onset and/or progression of OA, and consequently, single biomarkers diagnostics (to date) are not efficient enough to comprehensively classify all early stage OA patients of variable etiologies. Another explanation is that the change/delta within traditional biomarkers may be too subtle at the asymptomatic stage to efficiently distinguish patients from normal individuals (91),

and useful information can often be masked by the ‘noise’ generated by naturally occurring variation within a given population. Therefore, many groups have suggested that OA diagnosis should be considered in a more comprehensive manner (63).

Though systems biology is an effective technique for interrogating complex diseases, very few studies have been conducted within OA. Olex *et al*. integrated time-course microarray gene expression data from a mouse model into a protein-protein interaction (PPI) network (92). However, genomic responses in mice are much different than those in humans following an injury and concluded that mouse models might not be representative to study human inflammatory diseases (93). Nacher *et al*. applied a PageRank-based diffusion algorithm to recognize OA-related proteins in a chondrocyte protein network (94), and have suggested that protein Q6EEV6 could play a key role in OA development. In another similar study, some of the top hub genes PPI network are also among the differentially expressed genes, indicating that these genes may be the potential targets for OA diagnosis and treatment (95). All these studies share some common limitations. First, further genetic and experimental studies are needed to eliminate the possibility of false positive results from computational analysis. Second, all these studies are trying to find one or a handful of candidate biomarkers, which departs from the original purpose of the systems biology which is to undertake a more unbiased comprehensive approach to understand the complex intracellular and intercellular networks as a whole instead of simply measuring the values of single biomarkers. It is possible that these groups lacked effective methods to biologically interpret their network analysis results and therefore could only use a candidate

approach in their analysis (96).

Therefore, based on the current gaps of knowledge in the OA field regarding biomarkers in the earliest stages of OA, this thesis project was developed to fulfill the following objectives.

10.1 Objective

The overarching purpose of this thesis was to understand cytokine profiling in a number of distinct cohorts (including clinically diagnosed OA, high risk of OA and healthy controls) and potentially develop a biomarker indicative and possibly predictive of early stage (pre-radiographic) OA in a knee injury cohort.

Primary Objective: To determine if serum inflammatory profiles are different between the following groups: youth knee injury, clinically diagnosed knee OA, clinically diagnosed hip OA, and control groups (youth non-injured and older non-OA controls).

Hypothesis: Each group will have a unique inflammatory profile based on serum cytokine concentrations.

Exploratory Objectives 1: Apply data mining (develop a new mathematical approach if needed) on all available datasets to identify cytokines which could be potential biomarkers (e.g. significantly differentially expressed cytokines in high risk group; cytokines that are significantly correlated with pain, etc.) for early stage OA.

Exploratory Objectives 2: To validate the role of the most discriminative cytokine(s) from the patient cohorts in the onset/progression/mechanism of OA *in vitro* (using primary human cells) and *in vivo* (using an animal model of OA).

Chapter Two: Applying Computation Biology and 'Big Data' to Develop Multiplex Diagnostics for Complex Chronic Diseases Such as Osteoarthritis

2.1 Abstract

The data explosion in the last decade is revolutionizing the diagnostics research and the healthcare industry, offering both opportunities and challenges. These high-throughput "omics" techniques have generated more scientific data in the last few years than in the entire history of mankind. Here we present a brief summary of how "big data" has influenced early diagnosis of complex diseases. We will also review some of the most commonly used "omics" techniques and their applications in diagnostics. At last, we will discuss the issues brought by these new techniques when translating laboratory discoveries to clinical practices as well as several recent landmark cases in diagnostics industry.

2.2 Computational techniques in early diagnosis

The ability to provide effective treatments in early stage of a disease tends to lead to significantly better outcomes for the patient when compared to providing the same treatment at a significantly later stage of progression. This is particularly true for a number of diseases, such as cancer and cardiovascular diseases where any time lost can be a matter of life or death. However, early diagnosis of these diseases may be

challenging by traditional biochemical methods due to their asymptomatic nature and the lack of efficient detective technologies. In the last decade, an exponential increase in the amount of data has been produced by various high-throughput 'omic' technologies and we have now effectively entered the era of 'big data'. Though requiring massive computational resources and advanced data processing and analyses methods, 'big data' approaches to diagnostic medicine and biomarker development has had successes in the early detection of complex chronic diseases and in some cases has given us a deeper understanding of the molecular pathogenesis of disease. In this review we will discuss the current state of 'big data' and computational techniques for early stage disease diagnosis and how advances in these techniques may promote a better understanding of complexity diseases.

2.3 Big data in disease diagnosis

Although great progress has been made within the last few decades, classical biomedical research methodology is still facing a challenge against diagnosis of complex diseases which are typically associated with the effects of multiple genes in combination with lifestyle and environmental factors. One of the reasons for this difficulty in early diagnosis (or prediction) is that the changes of traditional biomarkers can be too subtle at the asymptomatic stage to efficiently distinguish patients from normal individuals (97), and useful information can often be masked by the 'noise' generated by naturally occurring variation within a given population. Therefore, many

groups have suggested that the diagnosis should be considered in a more comprehensive manner. Hampel *et al.* suggested a combination of multiple biomarkers as well as genetic predisposition and environmental factors should all be take into account for early diagnosis and personalized therapies of complex diseases such as Alzheimer's disease (98). However such studies require large-scale measurements on a large number of individuals to eliminate over-fitting of predictive models. With the development of high-throughput “omics” techniques and the reduction in prices per sample, now make these types of analyzes a reality. An enormous amount of data has been generated, providing a global view with rich information on diseases and their diagnosis. One of the largest projects is The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/), which contains clinical information, histopathology slide images and molecular information from over 8,000 tissue samples of 34 types of cancer. The goal of TCGA is to improve cancer early detection and treatment by understanding how DNA mutations interact to drive cancers. However, the interpretation of such rich information seems to be a “big data problem”. Big data is a concept that varies in different fields. In biomedical research, big data essentially refers to the computational analyses that help scientists make sense of the chaos of extremely large experimental and clinical data sets. Conceivably, big data is already impacting disease diagnosis. For example, by studying a large sample set, Chen *et al*. achieved considerable high specificity (98.9% and 91.9%) for non-invasive prenatal diagnosis of trisomy 13 and trisomy 18 using maternal plasma DNA sequencing (91).

Big data in disease diagnosis shares the same IT challenges in other fields, including

data storage, transfer, access control and management (99, 100). Another challenge is the computational modeling of complex biology systems. Due to the large scale and diversity of the data, un-optimized models may fall into NP hard (Non-deterministic Polynomial-time hard) problems whose time complexity increase super-exponentially (100), moreover, sampling bias should not be neglected. According to the study of Kaplan *et al.*, bigger data is not always better since large sample studies sometimes can actually magnify biases associated with error resulting from sampling or study design (101).

2.4 Computational "Omics" techniques

Diseases with an identifiable genetic component play a role in nine of the ten leading causes of death in the United States (102). A positive association between genetic variation and disease may not only help diagnose diseases at early stage, but also predict diseases before the initiation of pathogenesis. Genome-wide association study (GWAS) is one of the most common statistical approaches that involves rapidly scanning millions of markers (single-nucleotide polymorphisms, SNPs) at the same time across genome to find genetic variations associated with a common complex disease (103, 104). Liu *et al.* reported that the inclusion of the GWAS genetic variants data significantly improved their breast cancer naïve Bayes diagnostic model (105). As technological improvements continue to decrease DNA sequencing costs, whole genome sequencing (WGS) or whole exome sequencing (WES, sequence protein-

coding genes only) becomes more practical for clinical applications and might be a potential alternative to GWAS as it provides more information of whole genomes (106). However, WGS/WES generates large quantities of data which require tremendous computational analysis such as sequence alignment, variant calling, filtering and identifying disease susceptibility genes. In fact, sequence data is produced significantly faster than current computational resources can handle (107). Thus, more efficient algorithms and/or more powerful hardware need to be developed in the future (108). However, this may lead to an 'arms race' between hardware and software resulting in increased rates obsolesce in the field. Therefore, it is clear that both how the data is acquired (hardware) and how it is analyzed (software) cannot be pursued independently of each other.

Gene expression (transcriptomics) profiling provides an opportunity for accurate, definitive diagnosis (109, 110). High-throughput mRNA sequencing (RNA-Seq) is one of the most popular techniques in transcriptomics since this technology allows for investigating both known transcripts and uncovering new ones. Since transcripts (RNAs) need to be converted to cDNA and then sequenced, RNA sequence assembly algorithms for short, low-quality reads without references are required (111). While microarray suffers from a number of limitations compared to RNA-Seq (e.g., unbiased detection of transcripts, increased dynamic range, increased specificity/sensitivity, increased detection of rare/low-abundance transcripts) it can be used to measure large numbers of gene expression level simultaneously. In addition to regular clinical diagnosis, many recent articles reported the success of applying microarray in prenatal diagnosis (112,

113).

Proteomics can also be used for the biomarkers detection of early stage disease such as cancer (114, 115), cardiovascular disease (71, 116), Alzheimer's disease(117) and other chronic disease (118, 119). Mass spectrometers (MS) based proteomics can help the identification of all differentially expressed proteins and their post-translational modifications during disease progression which can be used as biomarkers for early diagnosis and monitoring disease treatment (77). The data process of MS relies heavily on open access public proteomics databases. Both our own group and others in the field have employed the use of high throughput ELISA technology such as Luminex and Meso Scale to examine panels of proteins (typically numbering between 20 and 60) in chronic diseases such as Osteoarthritis (120) and traumatic injuries (79).

Metabolomics, while a younger field than the rest, is rapidly expanding in the diagnostics field in "post genomic era". The metabolic characteristics and changes in the patients are influenced not only by which genes are transcribed, but also the composition of material that the cells obtain from their micro-environment. Many reviews have discussed the application of metabolomics in diagnostics using high-throughput techniques such as nuclear magnetic resonance spectroscopy (NMR) and MS. Madsen et al. made a comprehensive summary of metabolomics in cancer, diabetes, cardiovascular and other complex disease diagnosis (121). Zhang et al. pointed out that saliva metabolomics can be a potential method for personalized therapy and treatment monitoring (122). This being said however, data analysis is crucial for metabolomics based diagnosis, though in some cases, one single marker from the metabolic profile

might be sufficient to detect the diseases specifically, in most cases, machine learning techniques are applied to recognize and classify metabolic profiles or fingerprints between normal and disease states. Among them the most widely used are linear discriminant analysis (LDA), artificial neural networks (ANN) and support vector machines (SVM). Principal component analysis (PCA) is often employed for data dimension reduction before model training in order to lower the chance of over-fitting the model. Another way to avoid model over-fitting is to apply cross-validation techniques at the model training step.

2.5 System biology

Not until the completion of Human Genome Project was it realized that gene sequence alone was not sufficient to identify all the biology origin of disease. The function of each protein and the complexities of protein–protein interactions are critical for understanding physiological processes. In addition, recent studies shows that non-coding parts of the genome produce small conserved ribonucleic acids (non-coding RNA, ncRNA) which control molecular and cellular processes (123, 124). Thus, in order to develop effective diagnostic techniques and disease treatments, genomics, transcriptomics and proteomics should be studied integrally and systematically as a whole system.

Through a system-based approaches, Aldons et al. integrated genomic, molecular, physiological data with traditional genetic and biochemical methods to study complex

disease including diabetes and cardiovascular disease. He pointed out that analyzing the individual components of the whole system is far from sufficient, since in reality, these components interact with each other and these interactions play crucial roles in development of diseases (125).

A number of recent studies have successfully applied network models in describing and simplifying such complex systems (126-131). In these studies, network topology is used to investigate biological networks including metabolic networks, protein-protein interaction networks, gene regulatory networks, transcriptional profiling networks, etc., and their interactions. For example, in the gene network clusters created by Gilman *et al.* using NETBAG (network-based analysis of genetic associations), many proteins are found to participate in the formation of autism (130). These proteins may become new biomarkers for the diagnosis of autism. In another study conducted by Akutekwe *et al.*, a biomarker identification method using a dynamic Bayesian network to model the temporal relationship among the stratified features for early diagnosis of ovarian cancer (131). Matthias et al. tried to bridge the gap between genotype–phenotype by studying the inference of genetically perturbed molecular networks based on a combination of genomics, proteomics, and phenomics data (132). All these innovative strategies may provide us a deeper understanding of disease development and help us discover new indicators for early stage diseases.

2.6 Diagnosis of Osteoarthritis

Osteoarthritis (OA), one of the leading causes of chronic disability worldwide, is a form of arthritic disease which is characterized by the progressive destruction of articular cartilage. The pathogenesis of OA is multifactorial-aging, injury, genetic predisposition may all act as contributing factors which cause the joint cartilage degeneration. Currently, clinical diagnosis of OA relies on the radiographic assessment, pain symptoms and the mobility of the joint. Unfortunately, OA develops asymptomatically in early stage and when it becomes detectable, extensive and irreversible deterioration of joint has already occurred. Therefore, there is a need for new diagnosis methods, such as new specific biological markers, to detect OA at early stage before such deterioration happens. However, without understanding the biological mechanism of OA, the guideless search for effective biomarkers among billions of molecules for the early OA diagnosis is like finding a needle in a haystack. In the past few years, the development omics and bioinformatics techniques has impacted the etiology and diagnostics of complex disease like OA. High-throughput fast screening of biomarkers at whole omic level becomes a reality. Here we describe recent progress and challenge in OA diagnosis using these high-throughput techniques.

2.6.1 Genomics in OA diagnosis

GWAS have tested the association between thousands of SNPs in the whole genome and OA. So far, approx. 15 OA susceptibility loci have been identified by GWAS,

although some of them are gender or racial specific (133). Elliott *et al*. found significant overlap between OA and height and OA and body mass index (BMI) by comparing OA and BMI GWAS data, suggesting that OA and obesity may share genetic background (134). In a more comprehensive mate-analysis study, Rodriguez-Fontenla *et al*. summarized 9 GWAS of OA, they identified two genes (COL11A1 and VEGF) are significantly associated with hip OA development (135).

In order to find the rare variants that are missed in common GWAS studies, Boer *et al*. conducted a whole exome-sequencing study of 1,524 participants, in which 199 had hip OA. Besides 3 genes which are already identified in previous GWAS studies, they found gene FGF3 may contribute to hip OA by suppressing endochondral bone formation (136). Unfortunately, to our knowledge this is the only OA related whole genome/exome sequencing study published to date. To obtain a better understanding of genomic architecture of OA, additional whole genome large-scale NGS studies on various cohorts should be undertaken.

Several recent genome-wide DNA epigenetic studies using high-throughput arrays revealed new potential OA biomarkers. DNA methylation, one of the common DNA epigenetic modification at promoter region of the genomic DNA may influence DNA stability, chromatin structure and regulate the gene expression. Several studies have examined genome-wide DNA methylation profile of human articular chondrocytes in cartilage and trabecular bone samples from OA and healthy control to identify profiles of DNA methylation in OA disease (137, 138). They have all found significant differential methylation levels between the patient and normal groups and in the future

it is possible that these methylation sites and the genes which they are contained within could be used as new diagnostic markers.

2.6.2 Transcriptomics in OA diagnosis

Many microarray based gene expression studies on various tissue types from OA patients have identified differentially expressed genes and profiles which could contribute to the development of new biomarkers. For example, Blom *et al*. identified approximately 200 differential expressed genes (fold change $\geq \pm 2$) in synovium (139), whereas in peripheral blood, 86 genes were found expressed with at least 1.5-fold difference (140). As increased evidence indicates that the subchondral bone plays a major role in the initiation and progression of OA, Chou *et al*. performed a whole-genome gene expression study of subchondral bone (141). They found a total of 972 genes were differentially expressed (fold change $\geq \pm 2$) between normal and OA bone samples. Interestingly, however, between these studies, there are few differential expressed gene in common, suggesting that in OA, disease related gene expression change may be highly tissue and/or patient specific. Thought a few molecular models can explain a small portion of the tissue-dependent gene expression regulations, the full regulation mechanisms in different tissues are not clear (142). Nevertheless, it is essential that we consider the complex (and in some cases, non-canonical) roles of genes and their pathways in diverse tissue and cell types, and hence it is important to use expression data from the same tissues to maintain the comparability in order to

assess the association between genes and diseases in different studies.

2.6.3 Proteomics and metabolomics in OA diagnosis

Though proteomics and metabolomics approaches in OA diagnostic studies are relatively new, they have produced a great number of potential markers. A broad range investigation of proteomic profiles in different tissues has been conducted, including femoral head, humeral head, meniscus, explants, and etc. (80). Additional studies are more focused on human body fluid as the harvest is comparatively non-invasive and consequently will be easier to translate to clinical practices. Serum and urine are the most commonly used body fluids for proteomic analysis of OA (81). However, since they are spatially removed from the affected tissues it is possible that some key proteins may be diluted. Synovial fluid (SF), although sometimes difficult to obtain, can be studied as a compromise of non-invasiveness and sensitivity (143). A metabolomics analysis of synovial fluid has successfully classified the OA phenotypes into two metabolically distinct subgroups by the concentration of acylcarnitine which may be related to the carnitine metabolism pathway (144). These types of studies will help to unravel the complex pathogenesis of OA and simplify new biomarkers discovery by dividing OA into several subtypes.

A problem of current proteomics and metabolomics study of early OA diagnosis is that abnormal protein or metabolite expression are relatively dynamic comparing to gene mutations. Usually samples are obtained from patients who are already clinically

diagnosed with OA, therefore the proteomic and metabolomics profiles can only represent the status of the patients at the advanced or even end stage of the disease. Without knowing the changes of the profiles during OA progression, we should be careful to assume those differential expressed proteins or metabolite in late OA are the potential biomarkers for early OA diagnosis. Takinami *et al*. conducted a study which followed knee OA patients for 2 years to overcome this problem (81). However, OA is known to have a much longer pathogenic in some patients (even up to decades), and some evidence shows that cartilage degeneration which could ultimately lead to OA could start at youth. Therefore, it is essential to develop long term follow-up studies now, so that the next generation will be too able to benefit from these types of diagnostic studies in OA.

2.6.4 System Biology in OA diagnosis

Extensive “omic” data was screened so far and many biomarkers have been proposed, but their sensitivity or specificity is not high enough for clinical use and the reliability varies among studies (**Table 2-1**). One of the possible explanations for this is the multifactorial pathogenesis of OA, including aging, injury, and genetic predisposition may all act as contributing factors which cause OA, and consequently, single biomarkers diagnostics are not efficient enough to comprehensively classify all early stage OA patients of varies etiologies. Though system biology is an effective technique for complex disease search, very few studies have been conducted on OA. Olex *et al*.

integrated time-course microarray gene expression data from a mouse model into a PPI network (92). However, injuries can trigger very different responses in mice than in humans in terms of genomic responses, suggesting that mouse models might not be representative to study human inflammatory diseases (93). Nacher *et al*. applied a PageRank-based diffusion algorithm to recognize OA-related proteins in a chondrocyte protein network (94), they found protein Q6EEV6 could play a key role in OA development. In another similar study, some of the top hub genes PPI network are also among the differentially expressed genes, indicating that these genes may be the potential targets for OA diagnosis and treatment (95). All these studies share some common limitations. First, further genetic and experimental studies are needed to eliminate the possibility of false positive results from computational analysis. Second, all these studies are trying to find one or several biomarkers, which departs from the original purpose of the system biology study in complex disease-to study the complex intracellular and intercellular networks as a whole instead of simply measuring the values of single biomarkers. Lacking of effective methods to biologically interpreted network analysis results might be one of the reasons. Pilot works are needed to put into perspective computational analysis in the future.

Table 2-1. Biomarker approaches examined for the diagnosis of osteoarthritis.

Authors	Date	Biochemical Biomarkers	Body Fluid	Sample Size	Age	Gender	Comparison Group	Statistical Method	Sensitivity/Specificity
Singh et al. (53)	2014.12	Hyaluronic acid	serum	100/50 (cases/controls)	case51.28±7.93 control46.08±4.81 p<0.001	Case:34 males, 66females; Control:16 males 34 females	normal/mild OA	ROC curve	87.6%/86.0 %
Siebuhr et al. *(145)*	2014.01	hsCRP, CRPM	serum	12/202/57/60 (Mild/Moderate/Severe/TKR	mean:65.3	155 males 176 females	Mild/Moderate/Severe/TKR	ROC curve	33.3%/87.18%
Wisniewski et al. *(146)*	2014.02	TSG-6	synovial fluid, serum	91(OA)			high/low risk of rapid progression to end-stage OA	ROC curve	91%/82%
Heard et al. *(63)*	2014.06	38 cytokines	serum	100/100 (cases/controls)	normal40.0±9.5; OA60.4±10		normal/OA	ANN	100%/100%
Singh et al. (90)	2014.08	COMP	serum	100/50 (cases/controls)	Cases:58.93±9.20; control:54.68±9.68 p=0.01	50 males 100 females	normal/OA	ROC curve	98%/98%
Tanishi et al. *(147)*	2014.02	uCTX-II	urine	794/616 (OA/normal)	males: KL0,1:65.3±9.7 KL2:72.3±5.3 KL3:72.5±4.9 KL4:74.7±3.2 females KL0,1:60.7±9.5	435 males 605 females	KL0,1/KL>2	ROC curve	≈60%/40%men ≈70%/40%women （ROC curve）

					KL2:70.3±6.0 KL3:71.6±6.1 KL4:72.9±5.1"				
Zivanovic et al. *(89)*	2011.02	COMP	serum	66/22 (effusion/control)	69.97±9.37	20 males 68 females	with/without effusion(inflammation)	ROC curve	59%/50%
Han et al. *(148)*	2012.12	S100A12 and other two unknown proteins	synovial fluid	36/24(OA/RA)	OA:70.2±5.4; RA:68.4±4.9	OA: 9 males, 27 females RA: 4 males, 20 females	OA/RA	ANN	89.4%/91.2%
Henrotin et al. *(149)*	2012.07	Fib3-1, Fib3-2	urine	10/5 (cases/controls)	cases:76.0±5.0 control:25.6±2.6	15 females	normal/late OA	ROC curve	Fib3-1: 68.4%/77.1%, Fib3-2: 74.6%/85.7

2.7 Caution Ahead

Although these high throughput ‘omics’ platforms coupled with the application of complex bioinformatics approaches have had a number of successes in identifying potential biomarkers in complex diseases such as cancer (150, 151), sepsis (152), arthritis (63, 153) and others; it is important to realize that some, if not all complex diseases have numerous associated co-morbidities and risk factors. Therefore, it is essential to have extremely well characterized patient cohorts so that we can be sure we are not identifying biomarkers associated with those co-morbidities and/or risk factors. This is particularly important in diseases where no early diagnostic tests exist to assist in the confirmation/validation of the novel biomarkers.

2.8 Conclusion

The high-throughput “omics” techniques bring new energy to diagnostics, offering a comprehensive data resource from micro (e.g. genomics) to macro (e.g. phenomics). Facing the “big data” generated by such techniques, more powerful computational resources and efficient models or algorithms are needed for data storage, transferring and mining. System biology is one of the most successful theories that study the biology process by integrating multiple data resource. Plenty of studies have applied network models in describing etiopathogenesis and immune responses which may help discovery novel biomarkers for early diagnosis. However, we should be careful when apply those models especially when we are not sure if the clinical data is biased or not and no other diagnostic tests are available for validation. Last but not the least, the clinical translation should not be neglected. Though the diagnosis industry was frustrated by several case rulings which forbidden patents of genes and

biomarkers, novel diagnostic assay methods, kits, and method-of-treatment, or diagnostic methods combined with treatment programs using novel therapeutics may still remain patentable.

Chapter Three: Serum and Synovial Fluid Cytokine Profiling in Hip Osteoarthritis Patients: Distinct from Knee Osteoarthritis and Correlated with Pain

3.1 Abstract

Background: Inflammation is associated with the onset and progression of osteoarthritis in multiple joints. It is well known that mechanical properties differ between different joints, however, it remains unknown if the inflammatory process is similar/distinct in patients with hip vs. knee OA. Without complete understanding of the role of any specific cytokine in the inflammatory process, understanding the 'profile' of inflammation in a given patient population is an essential starting point. The aim of this study was to identify serum cytokine profiles in hip Osteoarthritis (OA), and investigate the association between cytokine concentrations and clinical measurements within this patient population and compare these findings to knee OA and healthy control cohorts.

Methods: In total, 250 serum samples (100 knee OA, 50 hip OA and 100 control) and 37 synovial fluid samples (8 knee OA, 14 hip OA and 15 control) were analyzed using a multiplex ELISA based approach. Synovial biopsies were also obtained and examined for specific cytokines. Pain, physical function and activity within the hip OA cohort were examined using the HOOS, SF-36, HHS and UCLA outcome measures.

Results: The three cohorts showed distinct serum cytokine profiles. EGF, FGF2, MCP3, MIP1α, and IL8 were differentially expressed between hip and knee OA cohorts; while FGF2, GRO, IL8, MCP1, and VEGF were differentially expressed between hip OA and control cohorts. Eotaxin, GRO, MCP1, MIP1β, VEGF were differentially expressed between knee OA and control cohorts. EGF, IL8, MCP1, MIP1β were differentially expressed in synovial fluid

from a sub-set of patients from each cohort. Specifically within the hip OA cohort, IL-6, MDC and IP10 were associated with pain and were also found to be present in synovial fluid and synovial membrane (except IL-6) of patients with hip OA.

Conclusion: OA may include different inflammatory subtypes according to affected joints and distinct inflammatory processes may drive OA in these joints. IL6, MDC and IP10 are associated with hip OA pain and these proteins may be able to provide additional information regarding pain in hip OA patients.

3.2 Introduction

Approximately 1 in 8 individuals are afflicted with Osteoarthritis (OA) and although it is more common in older populations, it is becoming a serious health and economical concern in young, active individuals (154, 155). Therefore, it is essential to further understand the factors involved in the onset and progression of the disease, so that more efficient diagnostics and treatments can be developed.

While much of the research focus in OA has been directed towards the knee, it is necessary to examine if the pathways and mechanisms in knee OA are conserved in other joints. For example, in the hip, recent literature have provided insight into mechanical causes of hip OA including impingement and dysplasia (156), while others have focused on potential genetic predispositions with or without mechanical risk factors (157). This suggests that, as in knee OA, there could be potentially many avenues of hip OA onset that eventually result in patients with diverse etiology converging later in the disease trajectory. Our previous work in knee OA looking at systemic and local inflammatory profiles suggests that inflammatory profiles are distinct in patients with knee OA from those without OA (63) and therefore that this approach may be able to discriminate systemic inflammatory differences between patients with knee vs. hip OA.

More generally, there have been a number of studies and many biochemical markers that have been identified in knee OA (82, 158-161). Historically groups have focused on cartilage metabolism markers including which demonstrate changes in concentration and fragment species with the onset and progression of OA (41, 162-164). Inflammation is known to be present in OA and the changes in individual inflammatory markers have been correlated with both severity and progression and OA (59, 165). Regardless of the type of biochemical marker examined however, knee OA and hip OA are almost always grouped together or only one joint is examined within a given study (166). Most of the common OA biochemical markers (collagen fragments, cartilage oligomeric matrix protein (COMP), etc.) have been tested on both knee and hip cohorts, but only a very few studies looked at both joints and run comparisons (166). The uncontrolled variances (such as different definition of OA, cohort characteristic, experimental method) across studies make the results almost incomparable. Among the studies that contain both cohorts, the comparisons of biochemical markers between knees and hips are largely ignored (167, 168). It is well known that mechanical properties differ between joints (knee vs. hip) (169, 170), but as far as we are aware only two studies have directly compared between cohorts of hip and knee OA patients: the effectiveness of bone metabolism for OA prognostic (171) and the difference of association between COMP and OA symptoms (172). However, neither of them compared the absolute concentration of biochemical markers between two phenotypes.

In addition to biochemical markers, it is also important to gain a better understanding of pain in OA. Pain is the leading disabling symptom of OA (173). According to the American College of Rheumatology (ACR) criteria for OA, pain is a necessary condition for clinical diagnosis of OA, with the support by other sufficient conditions such as age, radiographic evidences or physical examinations. OA pain is hard to quantify from patient to patient based on a number of confounding factors (174) and its inconsistency with other symptoms such as

radiographic findings is not yet well understood. Recent studies have greatly improved our understanding of pain at the molecular level (175), and new biochemical markers have also been reported to be correlated with severity of OA pain in the past decade (**Supplementary Table 9-1**). Cytokines play an important role in the pain signal pathway from lesions to higher brain processing centers (176). While cytokines can change the sensitivity of peripheral receptors to nociceptive input by a variety of mechanisms in experimental animal models (177-179), very few human studies have reported correlations between cytokines and OA pain. The chemokine sub-family of cytokines is also of specific interest in patients with arthritis since these small molecules are capable of recruiting additional immune and/or stem/progenitor cells to sites of injury and/or increased inflammation.

With our incomplete understanding of the role of any specific cytokine in the inflammatory process, understanding the 'profile' of inflammation in a given patient population is an essential starting point. Therefore, the primary aim of this study was to examine system and local inflammatory profiles in hip OA patients, using knee OA and non-OA control as comparator groups to identify if differences exist between hip and knee OA. Furthermore in the hip OA cohort, it was attempted to determine if there were any cytokine(s) that correlated with pain symptoms, as well as other clinical measurements.

3.3 Methods

3.3.1 Subjects and clinical evaluation

This study protocol was approved by the University of Calgary Human Research Ethics Board (REB15-0880). Participants (**Table 3-1**) provided written consent. In all patient cohorts a previous diagnosis of metabolic disease/disorder, diabetes and/or abdominal obesity excluded the individual from the current study. For all cohorts, prescription anti-inflammatory

medication use within the past three months also excluded the individual from the current study. Individuals from all cohorts presented in this study are a sub-set of a larger on-going cohort study at the University of Calgary and were selected based on the inclusion/exclusion criteria for this study. Sample sizes were based off our previous reported study [5].

Control (n = 100; mean age 40.0 ± 9.5 years): Individuals showed no clinical signs of OA or RA (based on ACR criteria), and were questioned (and potentially excluded) regarding personal (intraarticular joint injury, inflammatory arthritis, autoimmune diseases) and family (inflammatory arthritis including any autoimmune diseases) histories. Individuals (e.g. faculty, staff, students, volunteers) were recruited from the sports medicine program and human performance laboratory.

Knee Osteoarthritis (n = 100; mean age 60.4 ± 10 years): Inclusion criteria required a diagnosis of OA performed at the OA clinic (University of Calgary) based on clinical symptoms of 3 months or greater with radiographic (standing AP radiographs) evidence (Kellgren and Lawerence (K/L) grading: all radiographs were graded by a sports medicine physician and registered nurse first assistant: inter-reader reliability/kappa, $\kappa = 0.84$) of changes associated with OA in accordance with ACR criteria. A K/L grade of at least 1 was required for inclusion into the knee OA cohort. A previous diagnosis of hip, hand or spine OA resulted in exclusion and a clinical assessment was undertaken to identify potential undiagnosed OA in these joint was undertaken at the time of recruitment.

Hip Osteoarthritis (n=50; mean age 59 ± *9.5years):* Inclusion criteria was based on a diagnosis of hip OA determined by patient symptoms, clinical exam and radiographic (K/L) evidence (all radiographs were graded by an orthopedic surgeon and registered nurse first assistant: $\kappa = 0.87$) in accordance with ACR criteria. A K/L grade of at least 1 was required for inclusion into the hip OA cohort. A previous diagnosis of knee, hand or spine OA resulted in exclusion and a clinical assessment was undertaken to identify potential undiagnosed OA in

these joints was undertaken at the time of recruitment. Two major etiologies were impingement (n=35) and dysplasia (n=7).

Table 3-1. Demographics of 3 cohorts.

Characteristic	Knee OA (n=100)	Hip OA (n=50)	Control (n=100)
Gender (% female)	46	42	75
Age (SD)	60.2 (10.4)	59.0 (9.5)	40.0 (9.5)
K/L 0	0	0	100
K/L 1	72	0	0
K/L 2	1	0	0
K/L 3	28	14	0
K/L 4	0	36	0
Serum Sample	100	50	100
Synovial Fluid Sample	8	14	15*
Synovium Sample	5	5	5*

Footnote: *these sample are collected from cadaveric donations

3.3.2 Clinical Assessment of Hip OA Cohort

Patients were asked to consent to complete the following questionnaires at the time of surgery (Birmingham hip resurfacing or total hip replacement):

1. The Harris Hip score (HHS) (180). The score has a maximum of 100 points (best possible outcome) covering pain (1 item, 0–44 points), function (7 items, 0–47 points), absence of deformity (1 item, 4 points), and range of motion (2 items, 5 points).

2. Hip disability and osteoarthritis outcome score (HOOS) (181). It consists of 40 items assessing 5 subscales including pain, symptoms, activity limitations daily living, function in sport and recreation and hip related quality of life. Standardized answer options are given in 5 Likert-boxes with scores from 0 to 4 (no, mild, moderate, severe and extreme).

3. Short form 36 (SF36) (182). It consists 8 sections including vitality, physical functioning, bodily pain, general health perceptions, physical role functioning, emotional role functioning, social role functioning, mental health. Each measurement is scale from 0 (worst) to 100 (best).

4. The University of California Los Angeles (UCLA) activity score (183). The evaluation has

10 descriptive activity levels ranging from wholly inactive and dependent on others (level 1), to regular participation in impact sports (level 10).

The questionnaires were the most common ones in the literature concerning hip function and symptoms, and all of them had shown a good reliability.

3.3.3 Sample collection

Serum samples were collected at rest by standard venipuncture with untreated vacuum tubes. Serum was immediately aliquoted and stored at -80^{0}C until required for analysis. All samples analyzed were only thawed once (at the time of analysis).

Synovial fluid (knee and hip OA cohorts) was aspirated from the knee joint by the attending orthopedic surgeon or sports medicine physician. Control synovial fluid samples were obtained from the Southern Alberta Tissue Donation Program. Criteria for control cadaveric donations were an age of 40 years or older, no history of arthritis, joint injury or surgery (including visual inspection of the cartilage surfaces during recovery), no prescription anti-inflammatory medications, no co-morbidities (such as diabetes/cancer), and availability within 4 hrs of death. Synovial fluid samples were collected without the use of lavage. The samples centrifuged at 3000g for 15 minutes after blood has clotted at 4^{0}C and stored at -80^{0}C. All samples analyzed were only thawed once (at the time of analysis).

3.3.4 Synovium tissue collection

Control tissue samples were obtained from the Southern Alberta Tissue Donation Program based on the sample criteria for cadaveric donations as listed above. Synovial biopsies from OA subjects were taken during surgery.

3.3.5 Multiplexed arrays

Sample analysis was performed by Eve Technologies (Calgary, AB Canada) using the Milliplex MAP Human Cytokine/Chemokine Panel (Millipore), according to the manufacturer's instructions. All samples (serum and synovial fluid) were assayed at least in duplicate and prepared standards were included in all runs. The following proteins were examined by Luminex in this study: EGF, Eotaxin, FGF2, Flt3L, Fractalkine, GCSF, GMCSF, GROα, IFNα2, IFNγ, IL1α, IL1β, IL1rα, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12 (p40), IL12 (p70), IL13, IL15, IL17A, IL18, IP10, MCP1, MCP3, MDC, MIP1α, MIP1β, PDGFAA, PDGFAB/BB, RANTES, sCD40L, TGFα, TNFα, TNFβ, VEGFA. The sensitivities of these makers range from 0.1 – 10.1 pg/mL (average 2.359 pg/ml) and the inter-array accuracies range from 3.5% – 18.9% coefficient of variation (average 10.7%).

3.3.6 Immunohistochemistry

Immunofluorescence was performed on synovium specimens with fluorescent-conjugated antibodies were obtained as follows: PE Mouse Anti-Human MDC/CCL22 (Cat. No. 565950, BD), PE Mouse Anti-Human IP-10 (Cat. No. 555049, BD), FITC Rat Anti-Human IL-6 (Cat. No. 554544, BD).

3.3.7 Statistical methods

The normality of each cytokines was assessed by QQ plots. Because many of the cytokine concentrations were not normally distributed, both t-test and Mann–Whitney–Wilcoxon (MWW) tests were utilized. T-test and MWW were performed to compare the ages and BMI between groups. Principle component analysis (PCA) was used to reduce the dimensionality of the cytokine dataset and the first 3 components were used for 3-D scatter plot.

For hip OA cohort, MWW were used to compare the means of cytokines with pain scores,

BMI between two subgroups-impingement and dysplasia. PCA was run to extract two factors from 10 HOOS pain questions. Pairwise associations between cytokines and clinical questionnaire scores were assessed using Spearman's rank correlation test. First, we tested the correlation between 7 cytokines which have been reported correlated with osteoarthritis pain previously (176) and pain levels (evaluated by HOOS pain sub scores and SF36 body pain score). Then, we screened all the associations between every single cytokines and the log transformed ratios of any two cytokines and clinical variables (i.e., age, gender, BMI and clinical questionnaire scoring). P-values were adjusted by two different multiple testing correction procedures: Bonferroni procedure with familywise error rate of 0.05 ($\alpha \approx 0.001$) and Benjamini–Hochberg procedure with a false discovery rate of 0.20.

For both hip and knee OA cohorts, multivariable linear regression was applied to test the potential confounding effects including age, gender, K/L grade and affected joint (hip or knee) on cytokine profiles. Statistical analyses were performed using Python version 2.7 with scipy package (for screening associations between all cytokines and clinical questionnaire scores) and SPSS 20.0 (SPSS, Inc., Chicago IL). $P<0.05$ (two-sided) was considered statistically significant, except for the tests whose p-values were adjusted by multiple testing correction. The 3-D scatter plots of PCA were generated by SPSS 20.0. The bar graphs were generated by GraphPad Prism 6 (Graphpad Software. San Diego, CA).

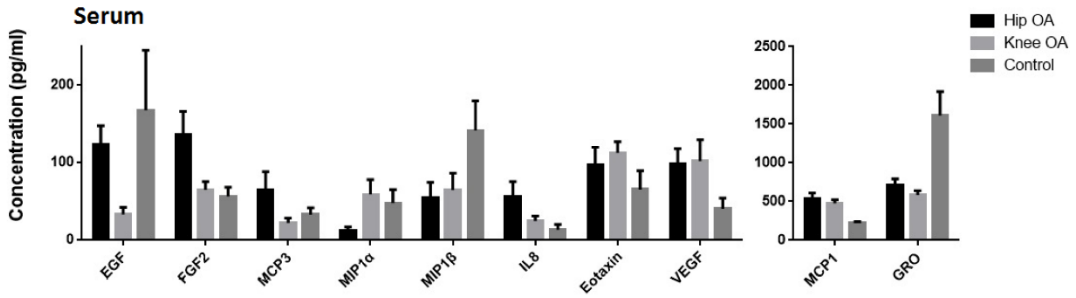
3.4 Results

3.4.1 Inflammatory Profile between Hip and Knee OA Cohorts

To uncover any differences in systemic inflammatory signaling in patients with hip OA vs knee OA or normal controls a multiplex approach was utilized in where we would quantify the levels of 42 cytokines (including lymphokines, interferons, colony stimulating factors and

chemokines, outlined in methods section) that are involved in many signaling pathways regulating inflammation and disease. Significant differences in serum cytokines levels were identified when comparing cohorts (hip OA vs. knee OA vs. control). While 5 cytokines were differentially expressed between knee and hip OA cohorts (**Figure 3-1**), 3 cytokines (GRO: growth-regulated oncogene aka C-X-C motif ligand 1/CXCL1, MCP1: monocyte chemoattractant protein aka C-C motif ligand 2/CCL2 and VEGF: vascular endothelial growth factor) were conserved in both hip and knee OA cohorts when compared to the control cohort (**Figure 3-1**). Overall, 10 cytokines were differentially expressed between all three cohorts. The first 3 components of PCA (explained 64.5% of total variance) clearly separated 3 cohorts (**Figure 3-1**).

A



B

	Hip OA	Knee OA
Knee OA	EGF*, FGF2, MCP3, MIP1α, IL8,	
Control	FGF2, GRO, IL8*, MCP1/CCL2, VEGF	Eotaxin, GRO, MCP1/CCL2*, MIP1β*, VEGF

C

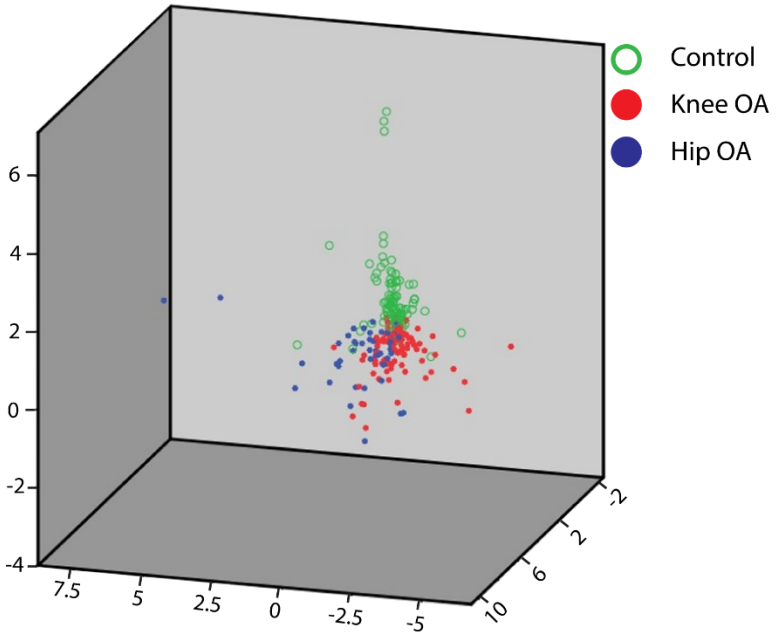


Figure 3-1. Comparison of cytokine profiles in serum in control, hip OA and knee OA cohorts. The serum concentrations of cytokines that were found to be significantly differently between the cohorts examined (a). The significantly different cytokines in each cohort are present in more detail in (b) after Bonferroni correction ($p < 0.0012$) (* = Significantly different in both serum and synovial fluid). Scatter plot of first 3 components of PCA of cytokine profiles of 3 cohorts demonstrates that the serum cytokine profiles are capable of discriminating the 3 different cohorts (c)

Synovial fluid cytokine profiles were also found to be distinct across 3 cohorts. Synovial fluid and serum shared common cytokines that were differentially expressed between cohorts: EGF (epidermal growth factor) differed between hip OA and knee OA; IL8 (interleukin 8) was different between hip OA and control; MCP1, MIP1β (macrophage inflammatory protein aka

C-C motif ligand 3/CCL3) differed between knee OA and control (**Figure 3-2**).

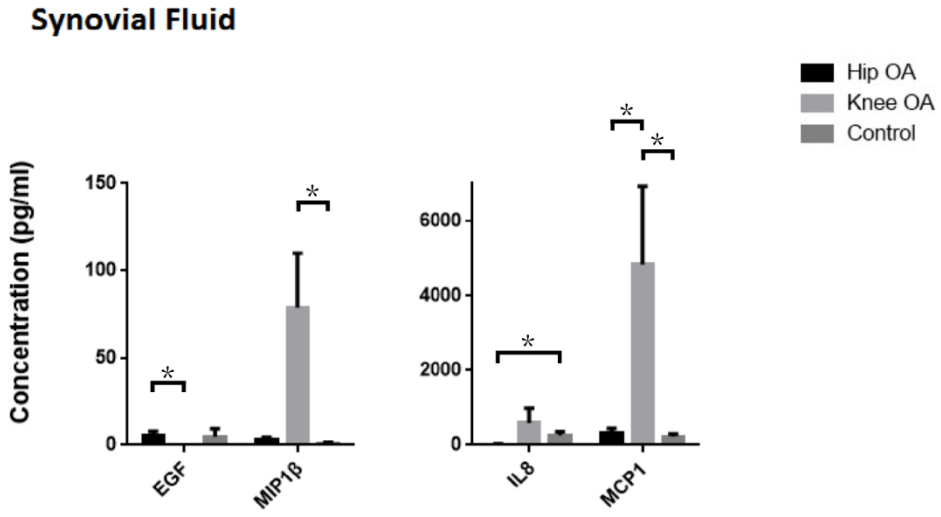


Figure 3-2. Differently expressed cytokines in synovial fluid in control, hip OA and knee OA cohorts. Of the cytokines examined in the serum that were significantly different between the hip OA, knee and control cohorts; EGF, MCP1, MIP1β and IL8 protein levels were also found to be significantly different between the synovial fluid samples obtained from the cohorts. Bonferroni correction (*$p < 0.001$)

Previous studies showed age, sex and severity of OA might have effects on cytokine expression (184, 185). Therefore, a multivariable linear regression model ($(y_{EGF} + y_{FGF2} + y_{MCP3} + y_{MIP1\boldsymbol{\alpha}} + y_{IL8}) = f(x_{age} + x_{gender} + x_{k/l\ grade} + x_{affected\ joint})$) was applied to access the correlations between cytokines and potential confounding variables (age, gender and K/L grade of OA and affected joint) in OA cohorts (**Table 3-1**). For cytokines that were significantly different between hip and knee OA cohorts, none were found to be related to age, gender or K/L grade, suggesting there were minimal effects of these variables confounding the cytokine profiles observed. After adjusting for confounding variables, EGF, FGF2 (fibroblast growth factor 2), MCP3 (aka C-C motif ligand 7/CCL7), and IL8 remained different between hip and knee OA.

Table 3-2. Multivariable linear regression result of associations between cytokines and

age, gender, K/L grade and affected joint (e.g. knee vs. hip).

Cytokines	Covariates	Sig.	95% CI	
			Lower	Upper
EGF	Age	0.396	-1.440	0.573
	Gender	0.177	-34.025	6.343
	K/L Grade	0.437	-7.964	18.331
	Affected Joint*	<0.001	-37.895	-14.098
FGF2	Age	0.370	-1.761	0.659
	Gender	0.195	-40.270	8.269
	K/L Grade	0.803	-13.809	17.809
	Affected Joint *	0.003	-36.048	-7.434
MCP3	Age	0.778	-0.751	1.001
	Gender	0.238	-28.085	7.046
	K/L Grade	0.909	-12.108	10.776
	Affected Joint *	0.005	-25.219	-4.508
MIP1α	Age	0.863	-1.426	1.197
	Gender	0.298	-40.207	12.400
	K/L Grade	0.572	-22.039	12.228
	Affected Joint	0.130	-3.555	27.458
IL8	Age	0.799	-0.881	0.680
	Gender	0.544	-20.479	10.832
	K/L Grade	0.731	-8.422	11.973
	Affected Joint *	0.049	-18.501	-0.042

3.4.2 Correlations between cytokines and Clinical Data in Hip OA Cohort

To determine if any clinical outcome measures were associated with the cytokine profile of hip OA patients, the pain, physical function and activity limitations of hip OA cohort were assessed using the HOOS, SF-36, HHS and UCLA scores.

First the discriminativity of all clinical measurements was examined by comparing each measurement between different severity of OA (different K/L grade). Six sub-scores showed a difference between K/L grade 3 and 4 groups (significant threshold was corrected by Benjamini–Hochberg procedure with a false discovery rate of 0.20) (**Supplementary Table 9-2**). These scores included the HOOS pain 5 (walking on a flat surface, p-value = 0.037),

HOOS pain 6 (going up or down stairs, p-value = 0.034), SF36 GH (general-health, p-value = 0.023), SF36 MH (mental health, p-value=0.007), SF36 PCS (physical component summary, p-value = 0.018) and SF36 MCS (mental component summary, p-value = 0.024). None were significant after Bonferroni testing correction.

Previously reported OA pain related cytokines (TNFα, IL1β, IL6, IL15, IL10, MCP1 and Fractalkine) (176) were also examined within our hip OA cohort dataset (**Supplementary Table 9-3**). Only IL6 was found to be significant when correlated with HOOS pain score factor 2 (correlation = 0.319, P-value = 0.024). When testing all 36 cytokines, 2 cytokines were found to be correlated with pain severity: MDC (macrophage-derived chemokine aka C-C motif chemokine 22/CCL22) was negatively correlated with BPSF36 (body pain SF36, correlation = -0.302, p-value = 0.033), IP10 was positively correlated with HOOS pain (correlation = 0.294, p-value = 0.038) and HOOS pain 8 (standing upright, correlation = 0.390, p-value = 0.005) (**Figure 3-3**).

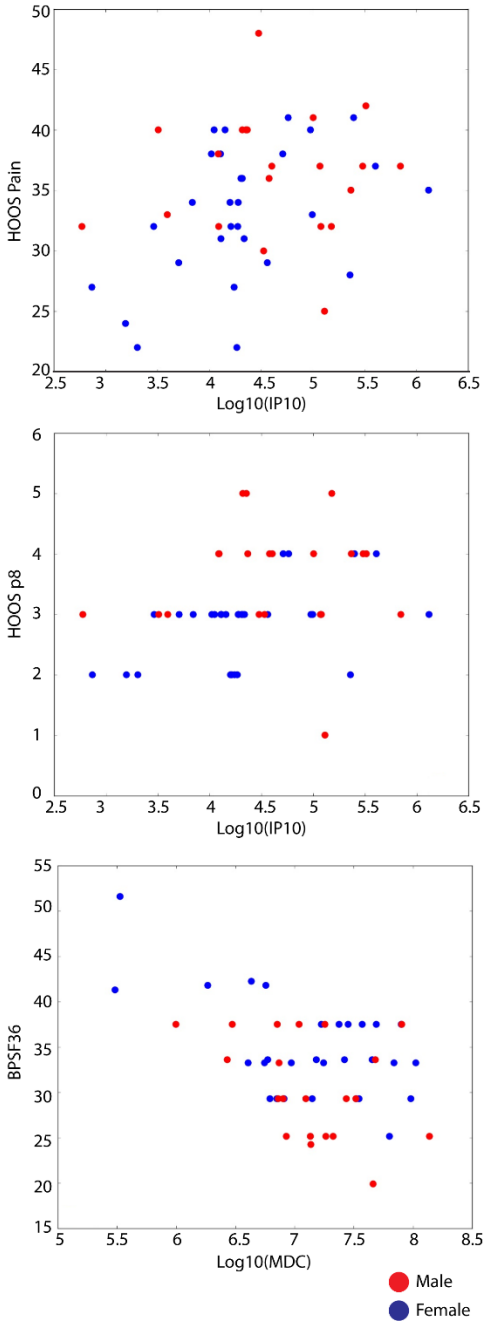


Figure 3-3. Scatter plot of two cytokines and correlated pain scores. Left: IP10 (log transferred) was positively correlated with HOOS pain score (correlation = 0.294, p-value = 0.038). Middle: IP10 (log transferred) was positively correlated with HOOS pain question 8 (correlation = 0.390, p-value = 0.005). Right: MDC (log transferred) was negatively correlated with SF36 body pain score (correlation = − 0.302, p-value = 0.033).

3.4.3 Synovium Examination from Control, Hip OA and Knee OA Cohorts

To determine if the cytokines that were correlated with hip pain could be originating from the joint environment, the expression of MDC, IL6 and IP10 were examined in synovial

membrane biopsies from a sub-set of the hip OA (n=5), knee OA (n=5) and control cohorts (n=5) (**Figure 3-4**). MDC and IP10 positive cells were observed in the synovium of patients with hip OA. IL6 positive cells were not regularly observed in the synovium of hip OA patients, but were observed in the synovium of patients with knee OA. MDC, IL6 or IP10 positive cells were not observed within the synovium of patients without OA. To further narrow down the potential source of the MDC, IL6 and IP10, synovial fluid and serum samples were examined in a sub-set of patients. High levels of MDC were observed in the serum of all cohorts, however, only patients with hip OA maintained high levels of MDC within their synovial fluid (**Figure 3-4**, **Supplementary Figure 9-1**). While IP10 was present in serum at lower levels, high levels were observed in the synovial fluid of hip OA patients alone. IL6 levels were present at low levels in serum and synovial fluid of all cohorts examined (**Figure 3-4**, **Supplementary Figure 9-1**). It is important to note that although multiple comparisons were not undertaken for this experiment, we chose to present the data (**Figure 3-4**) with the same multiple comparison correction as the previous results for consistency. However, a non-multiple comparison corrected version of the same data is presented in **Supplementary Figure 9-1**.

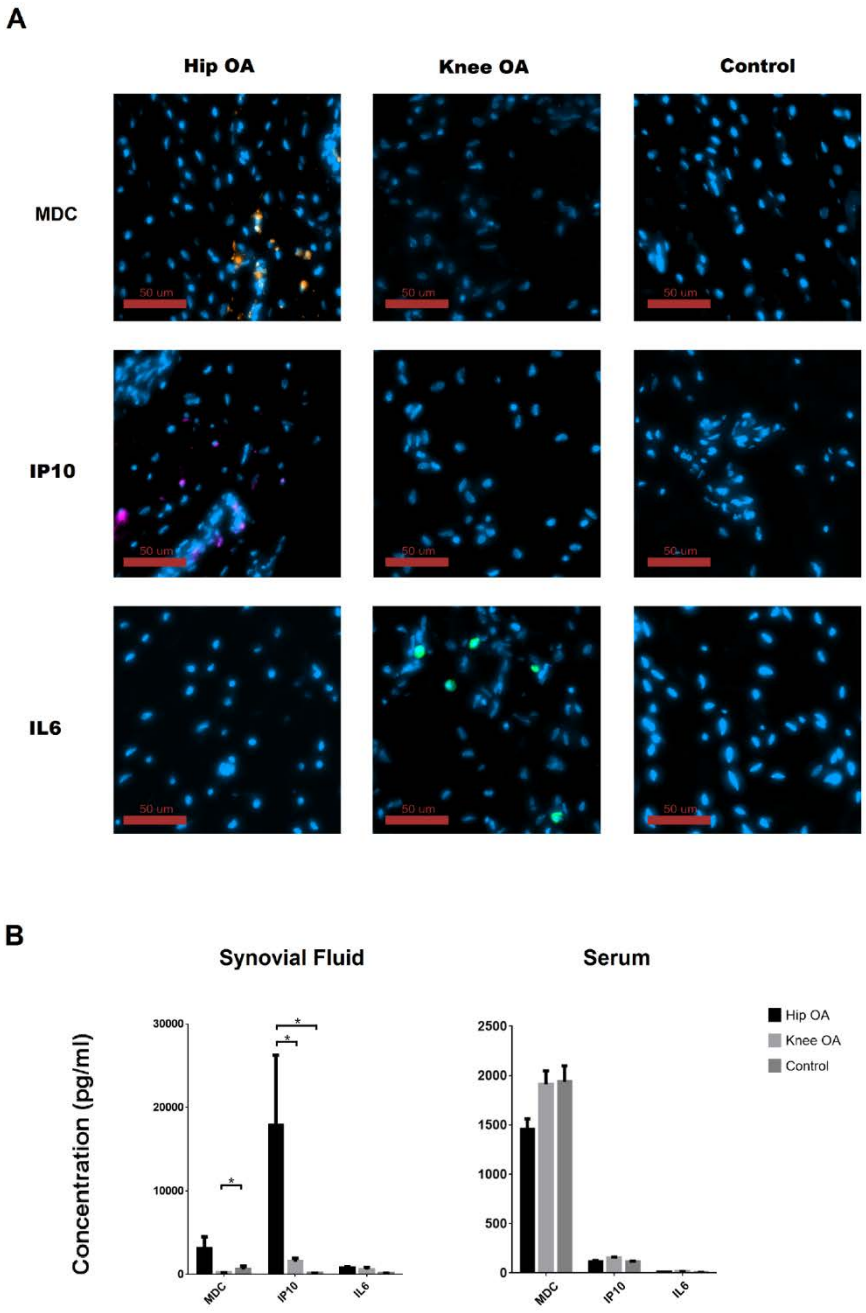


Figure 3-4. Presence of MDC, IL6 and IP10 in Hip OA, Knee OA and control synovium, synovial fluid and serum. Representative images demonstrating the presence of MDC (orange) positive (MDC: 3/5 hip OA, 0/5 knee OA, 0/5 control) and IP10 (purple) positive (3/5 hip OA, 0/5 knee OA, 0/5 control) cells in synovium of Hip OA patients. IL-6 (green) positive cells within the synovium were not observed in the Hip OA cohort, but observed with the knee OA cohort (1/5 hip OA, 4/5 knee OA, 0/5 control) (a). Serum and synovial fluid levels of MDC, IL6 and IP10 were also examined in the three cohorts (b). Bonferroni correction (*$p < 0.001$)

3.5 Discussion

To our knowledge, few papers have discriminated which joint the OA is affecting when comparing OA with control groups. In this study, we have found that 4 cytokines (EGF, FGF2, MCP3, IL8) are expressed differently in knee OA patients versus hip OA patients after controlling for age, gender and K/L grade. Interestingly, when we compared these two OA cohorts with the control cohort, the picture became even more complex. EGF levels were similar between the hip OA and control cohorts but both differed from the knee OA cohort. MCP3 was similar between the knee OA and control cohorts but differed from the hip OA cohort. The scatter plot of first 3 components of PCA of the 3 cohorts (hip OA, knee OA and control) showed that, neither Hip OA nor Knee OA is "closer" to the control. The center of gravity of the 3 cohorts represents a triangle shape (**Figure 3-1**). This infers that the three cohorts have their own characteristic cytokine profiles (though knee OA and hip OA cohorts are more similar to each other than either is to the control cohort). This may suggest that, at least for serum cytokines, OA may have distinct inflammatory profiles with subtypes that vary according to the affected joint. Failing to treat them as separate groups may be one of the reasons that the accuracy of biochemical markers in OA varies among studies (186). This may partially explain why none of the biochemical markers that have been studied to date have been approved for diagnosis or prognosis of OA. Another possible reason might be the heterogenetic and multifactorial nature of OA itself. The existence of subgroups in OA might invalidate any statistical single biochemical marker comparison which considers OA as one group-one biochemical marker is not enough to classify all of these subgroups. Previous studies have looked into subgrouping OA before statistical analyses (187). For instance, by using serum metabolomics data of OA patients, Zhang et al. identified at least 3 distinct subgroups which were not associated with any known confounders including age, sex, BMI and comorbidities (144). The current study suggests that hip and knee OA cohorts may not be as similar in terms

of the disease as previously thought and this has implications that OA in other joints (hands, spine, etc.) may also cloud diagnostic efforts, however, this hypothesis will require further study and validation.

In specific regards to the inflammatory cytokines examined in the current study, it is believed that aging brings about changes in the expression of many cytokines. Aged-related changes in the immune system may elevate the concentrations of cytokines which some have postulated may ultimately lead to chronic inflammation (188). This being said, in our dataset none of the cytokines are statistically significantly correlated with age. This might be due to the fact that most patients have passed their middle-age and the age distribution of these two cohorts are relatively concentrated (mean = 59.9, SD = 10.1). Although the control cohort is significant younger than the two OA cohorts (mean = 40.0, SD = 9.5). A limitation of the current study is that we unfortunately do not have the linked age information for each patient sample in the control cohort (only the cohort level details) and therefore we are not able to run a correlation analysis. Additionally, many factors can confound cytokine concentrations, such as statistical errors and mismatched cohort characteristics (such as races, disease history, diet and etc.). While, it can be unrealistic to find two exactly matched cohorts, these mismatch factors may lead to changes in serum cytokine concentrations in an unpredictable way and highly controlled cohort studies will be required to address these discrepancies.

TNFα, IL1, IL6 and IL17 have been reported to be correlated with development of neuropathic pain in various animal models (176, 189); however, except for IL6, which had weak correlation with PCA factor 2 of 10 HOOS pain questions, none of these proteins were significantly correlated with pain level scores in our dataset. We have tested the correlation between our two different pain level scores-SF36 body pain and HOOS pain sub-score, they are significantly correlated (correlation = 0.569, p-value < 0.0001). This suggests the method that we chose to evaluate osteoarthritis pain levels was reliable between questionnaires. Among

all 36 cytokines, we found that MDC and IP10 were correlated with pain in the hip OA cohort. IP10 (CXCL10) belongs to CXC chemokine family. It is expressed by a variety of cell types. When IP10 binds to CXCR3, this can result in activation and recruitment of leukocytes, and also regulate cell growth and apoptosis (190). In specific regard to pain, IP10 has been reported to be involved in breast cancer-induced bone pain by activation of microglia in rat models (191). In our study, IP10 was positively correlated with pain in patients with hip OA. It is possible that IP10 could be inducing OA pain through a similar pathway by activation of microglia in the joint environment. MDC (CCL22) belongs to the CC chemokine family and it is expressed mainly by macrophages and dendritic cells. MDC recruits Th2, Th17 and regulatory T cells through the binding of trafficking receptor CCR4 (192, 193). To our knowledge, no publication has directly looked at MDC and pain.

The concentrations of these cytokines in synovial fluid were consistent with the immuno-histochemistry results; with MDC and IP10 higher in synovial fluid than in serum, and were higher in hip OA than knee OA or control cohorts. This strongly suggests that within the hip OA cohort, MDC and IP10 are being produced within the joint environment and then may diffuse into serum through the synovial fluid. In the knee OA cohort however, MDC and IP10 were not present within the joint (synovium or synovial fluid) and therefore any detectable levels of these in the serum are most likely being generated in other tissues in the body. While, the concentration of IL6 was higher in hip and knee OA cohorts than controls, IL6 was not found in hip synovium. This suggests that there might be other tissues in the joint (cartilage, ligaments, etc) that produce IL6 in the hip OA cohort. The relationship between serum and synovial fluid cytokine levels are still poorly understood with previous studies demonstrating little correlation (194, 195) or high correlation (194, 195) between systemic vs. local levels. However, the biological basis behind these correlations (or lack thereof) remain unknown, and

further study will be required on specific markers to determine if the choice of sample local will impact the usefulness of the result obtained.

3.6 Conclusions

These findings validate previous studies that cytokines are differently expressed in OA patients (165); and suggest that while cytokine profiles are generally similar between hip and knee OA, that there are specific differences that may be related to differential disease processes within a given joint. Furthermore, 3 cytokines (IL6, MDC and IP10) were identified that correlated with hip OA. These results set the ground work for future studies that will be required to understand: if cytokines play distinct roles in in the onset/pathogenesis of OA in different joints; and what is the pathway and/or mechanism by which the identified cytokines regulate OA pain.

Chapter Four: Biochemical Markers for the Early Identification of Osteoarthritis: Systematic Review and Meta-Analysis

4.1 Abstract

Introduction:

Osteoarthritis (OA) is a major cause of disability in elderly populations and a huge burden of healthcare systems in western countries. There is a desperate need for the efficient and reliable detection of OA at the early stage when patients are likely to benefit most from disease interventions. In the past decades, a variety of biochemical markers from diverse bodily fluids have been investigated. However, their reliability varies among studies. In this review, we aim to answer following questions: 1) Are there biochemical markers that differential expressed in early OA vs. healthy subjects, and 2) If so, what is the diagnostic value of these biomarkers for early OA.

Methods:

EMBASE, PubMed, and Web of Science were searched to obtain all relevant studies up to March 2018. The Down and Black checklist was used to assess for bias. Biomarkers that were investigated in 5 or more different populations were pooled for meta-analysis. A meta-regression analysis was performed to explore the possible explanations of the heterogeneity for studies.

Results:

Twenty five articles met the criteria for the qualitative synthesis and seventeen articles

(included 3 biomarkers) for the final quantitative synthesis. N-Terminal Crosslinked Telopeptide of Type I Collagen (NTX-I) was the only biomarker found to be differently expressed in early OA patients vs. controls without significant heterogeneity among studies (I^2 = 0 %, χ^2_4 = 1.695, P = 0.792). The meta-regression analysis identified sample size and affected joint which might explain the heterogeneity among studies.

Conclusions:

Although a wide range of biomarkers have been investigated, the diagnostic value of these biomarkers cannot be determined due to the small number of studies. Large prospective and adequately powered studies are still needed to validate the role of these and other biomarkers for identifying early OA.

4.2 Introduction

Osteoarthritis (OA) is a chronic disease and major cause of disability in elderly populations (196, 197). According to World Health Organization, by 2050, 130 million people worldwide will suffer from OA and one-third of whom will be severely disabled by the disease (196). It is estimated that the economic cost of OA could be up to 2.5% of the gross domestic product (GDP) for westernized countries place more strain on already burden health care systems (198). OA is typically characterized by the progressive loss of articular cartilage with changes in other joint tissues such as subchondral bone and synovium. Current clinical diagnosis of OA heavily relies on symptoms (e.g. pain, swelling, stiffness) with the support of radiographic assessment

(cartilage loss, joint space narrowing and osteophytes) (19). Therefore, it is common for OA patients to seek medical consultation only when symptoms appear and/or when these symptoms become difficult to manage independently. Consequently, in the vast majority of the population, OA is only detected at advanced stages when significant, and possibly irreversible damage within the joint has already occurred. This issue is compounded by the fact that no non-surgical treatments have been approved by the FDA that can slow, stop or reverse the progression of OA (161). This typically results in patients suffering from OA undergoing symptom management until the patient is a candidate for joint arthroplasty, and even though this procedure is quite effective, and the overall patient satisfaction rate is variable.

The American College of Rheumatology (ACR) criteria for the clinical diagnosis of knee OA was first published in 1986 (64), followed by hand and hip OA in 1990 (199) and 1991 (65). It is still the most widely accepted and clinically applied OA identification criteria worldwide. However, a reliable identification method of early OA is still lacking. Reasons behind this are that symptoms in the early stages can be sporadic/intermittent and structural changes within the joint are minimal and therefore it is difficult to detect non-invasively. Furthermore, not all sub-types of OA present with the same risk-factors and/or rate of progression. Despite these difficulties and others, there is a desperate need for the efficient and reliable detection of early OA. Because at the very least, patients are likely to benefit most from disease interventions in the earliest stages of the disease. Biochemical biomarkers are an ideal candidate for this asymptomatic early-stage OA as they may directly or indirectly participate in the

biochemical processes of onset and/or progression of the disease. In the past decades, despite the slow development of the understanding of the complex biological nature of OA, a variety of biochemical markers from diverse bodily fluids have been investigated. However, their reliability varies among studies. In this systematic review, we aim to summarize previous human studies of biochemical markers and address the following questions: 1) Are there biochemical markers that are differentially expressed in early OA vs. healthy subjects, and 2) If there are any biochemical markers differentially expressed between early OA vs. healthy subjects, what is the diagnostic value of these biomarkers for early OA.

4.3 Methods

This systematic review and meta-analysis were conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (http://www.prisma-statement.org).

4.3.1 Search strategy

A comprehensive literature search was undertaken using EMBASE, PubMed, and Web of Science to obtain all relevant studies up to March 2018. In order to ensure inclusion of all available publications, the primary screening was split into 2 steps. First, a broad search was applied using the following search term: “early”[All Fields] AND “osteoarthritis”[All Fields]. Retrieved references were organized using EndNote X8.

Secondly, “marker” [All Fields] was used to refine our search using the search function in EndNote X8. Since the search in EndNote x8 was a simple “string match”, which finds all words contains the pattern “marker” (e.g. biomarker, biomarkers, bio-marker, biochemical-marker). Therefore, all possible derivations of “biomarker” were included. Titles and abstracts were then manually screened for eligibility, and potentially relevant studies were selected for full-text reading. Full texts were considered relevant and were selected for qualitative synthesis if met the following eligibility criteria.

4.3.2 Eligibility criteria

The diverse interpretations of “early OA” among researchers made it difficult to establish criteria for screening literature in this review. The majority of literature interpreted “early OA” as the “early stage” after clinically established OA, while the remainder followed a definition that early OA should be based on the fact that the patient cannot be classified as established OA (200). Therefore, we developed criteria 4(a) and 4(b) to include both types of literature.

Publications were therefore included for full-text review if they meet the following criteria: 1.) Evaluated biochemical marker(s); 2.) Included human subjects; 3.) The full text was available and in English, and; 4.) one of the following criteria: a.) case or cohort control study with both early OA* patients and non-OA participants as controls. b.) Longitudinal study included “non-OA” or “early-OA*” participants at baseline with the incidence of OA at follow up within 10 years

*: Early OA is defined by the authors (**Table 4-1**)

The exclusion criteria for meta-analysis were as follows: 1.) Data was judged to be inconsistent or demonstrated to be invalid based on further research; 2.) Data format was unavailable for meta-analysis; 3.) a newer publication reported the same biomarker results from the same study population.

4.3.3 Data extraction

Data extraction was performed on published data only. The following information was extracted for qualitative synthesis: authors, publication year, race/country of subjects, gender, age, sample size, the OA affect joint of interest/study, biochemical biomarker(s) investigated, the fluid/tissue specimen from which the biomarkers were obtained, the experimental methodology used for measuring the biomarker(s). A study was included in the qualitative synthesis (meta-analysis) if it contained at least one of the following data: 1.) The mean and the standard deviation of the concentration of the biomarker in both early OA and a control population; 2.) The odds ratio and its confidence interval (CI) of the biomarker between early OA and a control population; 3.) Student's t-test p-value for the biomarker between early OA and a control population; 4.) Data that can construct a 2×2 table (e.g. number of false positives, false negatives, true positives and true negatives, or the sensitivity and specificity or receiver operating characteristic (ROC)).

4.3.4 Risk of bias assessment

The Down and Black checklist was used to assess the study quality regarding the risk of bias. Items related to an intervention were excluded (Items 4, 8, 12, 13, 14, 15, 19, 23, 24). Item related to follow-ups (9, 17, 26) were excluded for cross-sectional studies. Therefore, the risk of bias was evaluated on the remaining 18 items for longitudinal studies and 15 items for cross-sectional studies.

4.3.5 Statistical analysis

R version 3.5.0 with metafor package was used to perform all statistical analyses. Where possible, data from studies looking at the same biomarker were pooled to conduct a meta-analysis. Data were normalized by calculating the standardized mean difference (SMD, Cohen′s *d*), with 95% confidence intervals (CI) as an effect size (ES) estimate. The SMD for each biomarker was pooled using a random-effects model. According to Cohen, the magnitude of the SMD was considered small if SMD = 0.2; medium if SMD = 0.5; and large if SMD = 0.8. In order to maintain sufficient statistical power and to avoid bias, only the SMD for the biomarkers that were investigated in 5 or more different populations were pooled for meta-analysis. Between-study heterogeneity was assessed using the I^2 statistic and the χ^2 test. Meta-regression analysis was performed to explore if participant characteristics could explain the heterogeneity for all studies. The covariates that were evaluated included sample size, the age difference between early OA and control group, sex, and affected joint using random effects models (restricted maximum likelihood estimators).

4.4 Results

A total of 15,741 articles were obtained by the first step of the primary search. Eight hundred forty-five, 7351, and 7555 articles were retrieved from the Embase, Pubmed, and Web of Science databases, respectively. After the second step of the primary search, 125, 645, and 703 articles from the 3 databases remained. Then 248 duplicates were removed, the remaining 1225 articles were screened for titles and abstracts and 147 articles were selected for full-text review. 25 articles met the criteria for the qualitative synthesis and 17 articles for the final quantitative synthesis (**Figure 4-1**). The characteristics of the studies included in the final quantitative assessment are shown in **Table 4-1**.

Figure 4-1. Flow chart of the study protocol

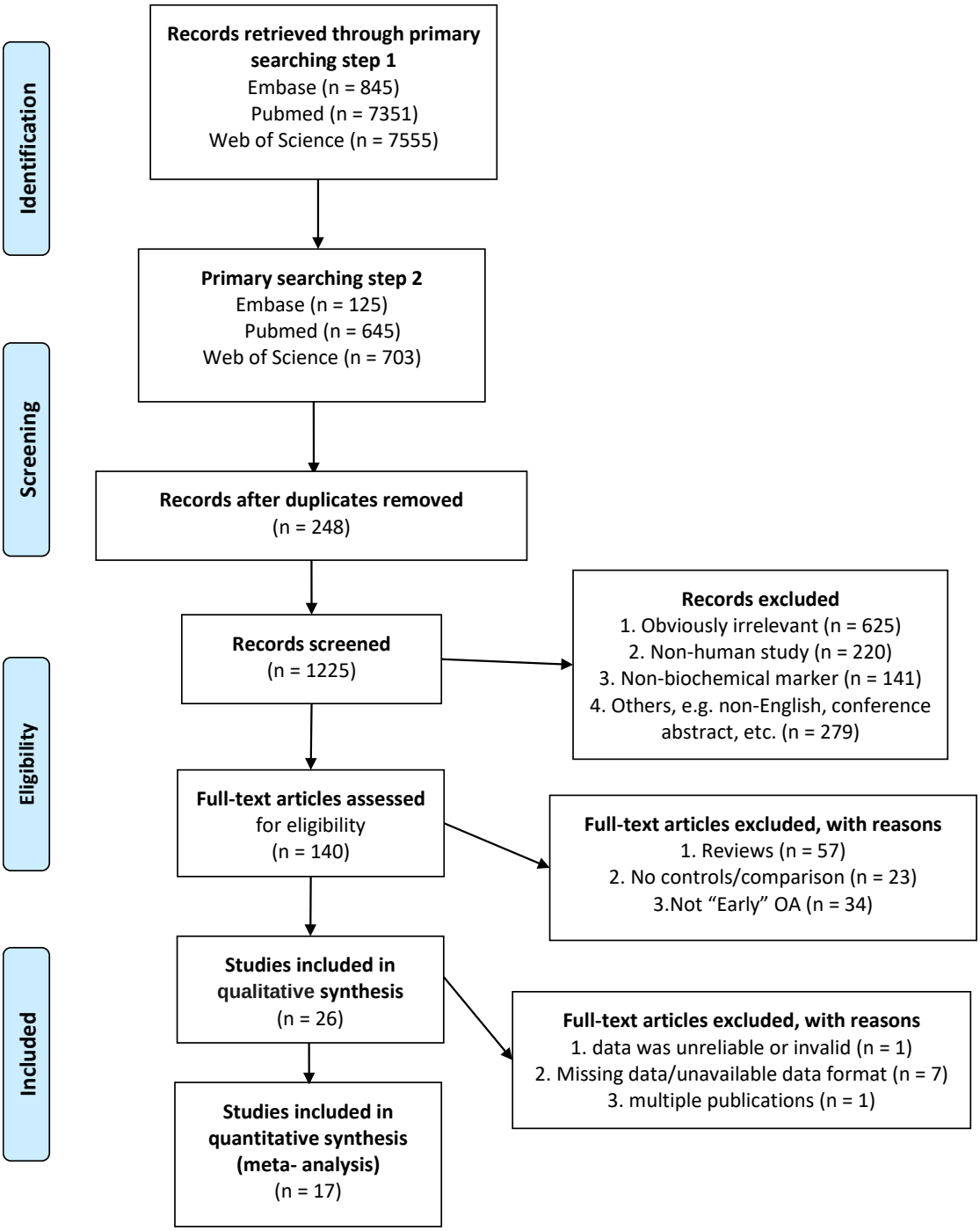


Table 4-1. Characteristics of included studies in the qualitative analysis.

ID	Author Year	Ethnicity / Country	Study design	No. of case /control	Early OA Definition	Sex (Female %)	Age (baseline in longitudinal study)	Biomarker(s) Studied	Specimen	Methodology	Joint	Sensitivity /Specificity	Case vs. Control statistic (significant only)
1	Petersson 1995 (201)	Sweden	longitudinal study	23/15	Incident of radiographic OA at follow up	44.7%	47 (range 37-54)	COMP,BSP	Serum	ELISA	Knee		δCOMP: p=.002, δBSO: p=.004
2	Zhang 2015 (202)	China	Cross-sectional cohort control	20/20	K/L grade ≤ 3	50%	Control: 56.3 ± 7.9, Early OA: 59.2 ± 8.3	CRP, WBC, Creatinine	Serum	UPLC-MS	Knee		p=.02
3	Zhang 2014 (203)	China	Cross-sectional cohort control	50/25	Outerbridge grade ≤ 2	51.2%*	40.5 ± 15*	Ihh	SF	ELISA	Knee		p=0.008
4	Xin 2017 (204)	China	Cross-sectional cohort control	19/20	K/L grade = 1	77%	Control: 57.6± 6.1, Early OA: 56.7 ± 6.9	CTX-II, Zn^{2+}, Ca^{2+}	Urine	ELISA, ICP-AES	Knee		Zn^{2+}: p<.05, Ca^{2+}: p<0.5
5	Wakitani 2007 (205)	Japan	Cross-sectional cohort control	7/24	K/L grade ≤ 1	56.8%*	range 20–80*	KS, C6S, CS846, HA and COMP	Serum	ELISA, HPLC	Knee		KS: p<.05, HA: p<.05 COMP: p<.05
6	Van Spil 2015 (206)	The Netherlands	longitudinal study	Knee: 132/298, Hip: 68/477	K/L grade ≤ 2	79.0%	56 ± 5	CTX-II, COMP, PIIANP, CS846, CTX-1, NTX-1, PINP, OC, HA, PIIINP	Serum & urine	ELISA	Knee & hip		p=0.033
7	Sugiyama 2003 (207)	Japan	longitudinal study	112/112(follow up/baseline)	Incident of radiographic OA at follow up	100%	50.2 ± 6.0	PIICP	SF	ELISA	Knee		
8	Steinbeck 2007 (208)	USA	Cross-sectional cohort control	11/4	mild arthritic symptoms	N/A	Control: 43 ± 9.6, Early OA: 61 ± 17.4	MPO	SF	ELISA	Knee		p<.005

9	Poole 2016 (209)	Canada	longitudinal study	120.1/34.4 (weighted)	MR cartilage scores > 2 & K/L grade < 2	Control: 58%, early OA: 51.4~~%~~	Control: 49.5 ± 9.9, early OA: 54.9 ± 9.4	C2C	urine	ELISA	Knee		p=.029
10	Marshall 2005 (210)	Canada	Cross-sectional cohort control	84/76	K/L grade ≤ 2	Control: 39.4%, early OA: 40.5%	Control: 36.9 (range 20-67), early OA: 31.9 (range 20-67)	combination of 8 genes	Serum	DNA microarray	Knee	76%/78%	
11	Kosinska 2014 (211)	Germany	Cross-sectional cohort control	17/9	Outerbridge grade ≤ 2	Control: 12.5%, early OA: 35.3%	Control: 22 (range 21–25), early OA: 36 (25–49)	a variety of sphingomyelins, hexosylceramides and rdihexosylceramides	SF	ESI-MS/MS	Knee		p<.05
12	Kobayashi 1997 (212)	Japan	Cross-sectional cohort control	50/31	radiological evaluation grade I	83.1%	68, 42–88 (median, range)	procollagen II C-propeptide	SF	ELISAS	Knee		
13	Jiao 2016 (213)	China	Cross-sectional cohort control	34/14	Outerbridge grade ≤ 2	54.4%*	42.5±16.6 (mean+sd)*	COMP, HA, CTX-II, MMP-3, PIINP	Serum	ELISAS	Knee		
14	de Seny 2011 (214)	Belgium	Cross-sectional cohort control	68/36	K/L grade = 0	Control: 55.5%, early OA: 69%	Control: range 47-64, early OA: range 37-79	V65 vitronectin fragment, C3f, m/z value 3762, CTAPIII	Serum	SELDI-TOF-MS	Knee		C3f: p<.001
15	Bassiouni 2011 (215)	Egypt	Cross-sectional match sex control	10/15	K/L grade = 1	58%*	Control: range 20-43, early OA: range 40-75	TIMP-1, MMP-3	Serum	ELISA	Knee		
16	Ahmed 2015 (216)	UK	Cross-sectional cohort control	30/37	Outerbridge grade ≤ 2&	N/A	N/A	multiple protein adducts	Serum	LC-MS/MS	Knee	0.73/0.87	

					normal radiographs of the symptomatic knee							
17	Vos 2013 (217)	The Netherlands	longitudinal study	183/183(follow up/baseline)	sum K/L grade < 4	83.6%	55.5 ± 5.4	pentosidine, CTX-II	Skin & urine	HPLC, ELISA	Knee	uCTX-II: p=.001
18	Ling 2009 (218)	96% Caucasian, USA	longitudinal study	66/22	K/L grade ≤ 1	Control: 59.0%, early OA: 57.1%	Control: 52.3±14.4 (median), early OA: 58.0±15.2 (median)	combination of 16 proteins	Serum	RCA & microarray	Knee	
19	Wei 2014 (219)	China	Cross-sectional cohort control	8/7	Non-clinically diagnosed OA	100%	Control: 49 (mean), early OA: 73.6 (mean)	Aggrecan, COL2A1, COL1A1, COL10A1	Cartilage	qRT-PCR	Hip	
20	Livshits 2009 (220)	UK	longitudinal study	1,003 (cohort size)	K/L grade ≤ 1	100%	45–64	IL-7, TNFα, CRP	Serum	ELISA	Knee	
21	Kumm 2013 (221)	Estonia	longitudinal study	161 (cohort size)	K/L < 1	66%	45.0 ± 6.2	PINP, OC, MidOC	Skin & urine	ELISA	Knee	
22	Chaganti 2008 (222)	Caucasian, USA	longitudinal study	167/169	Incident of radiographic OA at follow up	100%	Control: 69.6 ± 3.7, early OA: 70.8 ± 4.6	COMP, NTX-I	Serum	ELISA	Hip	
23	Sowers 1999 (223)	USA	longitudinal study	9/473	K/L grade ≤ 1	100%	37.4 ± 4.8	Osteocalcin	Serum	Radioimmunoassay	Knee	
24	Kelman 2006 (224)	Caucasian, USA	longitudinal study	COMP: 186/194, NTX: 198/199	Incident of radiographic OA at follow up	100%	Control: 67.9 ± 3.8, early OA: 70.9 ± 4.6	COMP, NTX-I	Serum	ELISA	Hip	COMP: p<.05, NTX: p<.05

25	Gebhard 2003 (225)	Germany	Cross-sectional cohort control	21/21	Mankin's grades 3-5	N/A	Control: 56 (mean) 39-76 (range), early OA: 65 (mean) 39-91 (range)	COL1, COL2, COL2A, COL3, COL10	Cartilage	qRT-PCR	Knee	
26	Cibere 2009 (226)	Canada	Cross-sectional cohort control	105/16	MR cartilage scores ≧ 1 & K/L grade ≤ 1	Control: 50.0%, early OA: 52.4%	Control: 46.4 (median), early OA: 55.2 (median)	CTX-II, C2C, C1,2C, CPII, NTX-I, CS846, COMP, HA	Serum & urine	ELISA	Knee	uC2C: OR 2.06, 95% CI (1.05–4.01), uC1,2C: OR 2.07, 95% CI (1.12–3.77)

4.4.1 Characteristics of included studies

Among 26 publications, 11 were longitudinal studies and 15 were cross-sectional cohort/case-control studies. The definition of early OA varied among studies. Radiographs with K&L classification were the most common non-invasive method to quantify damage of OA within the joint. Four studies directly visualized (arthroscopy) and measured the damage in the cartilage and quantified with Outerbridge grading. The majority of publications (24 out of 26) investigated early OA in the knee joint, which included ~1369 early knee OA cases and ~1194 controls. Three publications examined hip joints, including 68 early hip OA cases and 653 controls. The average female percentage of all subjects was ~68.62%, with 6 studies including only female participants. The remained of the studies included both male and female subjects with female participant percentage ranging from 12.5% to 81.3%. Few studies reported the ethnicity details of the study subjects. However, since none of the studies reported ethnicity selection biases, we have made the assumption that the ethnicity of subjects was similar to the local population from where the studies undertook. A wide range of biomarkers was evaluated, including metabolisms, cytokines, metal ions, gene expressions and unknown proteins identified through mass spectrometry. The biomarkers in the studies selected were identified primarily from: serum, urine and/or synovial fluid. Two studies investigated biomarkers in the cartilage which were harvested from different sources including OA patients that underwent total joint arthroplasty, patients with a femoral neck fracture and healthy donors.

4.4.2 Quality assessment

The methodological quality scores of the studies are presented in **Supplementary Table 9-4**. The scores ranged from 66.7% to 100%, with higher scores indicating higher quality assessment. While the majority of studies selected for analysis demonstrated adequate or above quality; in some studies, the confounders were not investigated, or were identified but without addressment in the statistical analysis. Furthermore, it was found in small number of studies examined that sample size had compromised the statistical power to detect a clinically important effect.

4.4.3 Analysis of biomarkers

In a total of 35 biomarkers were included in the 17 studies which qualified for quantitative synthesis. Unfortunately, most of the biomarkers in the selected studies were only examined in 1 or 2 manuscripts. Three biomarkers were investigated in more than 5 different populations, these included: Cartilage oligomeric matrix protein (COMP), Hyaluronic acid (HA) and N-Terminal Crosslinked Telopeptide of Type I Collagen (NTX-I).

Seven studies assessed the COMP levels in serum. Of these, 4 were longitudinal and 3 were cross-sectional. Five of these studies included both males and females, 2 consisted entirely of women. The overall SMD for COMP was small 0.128 (95% CI -0.102 to

0.358). Between-study heterogeneity was found among studies (I^2 = 82.96 %, χ^2_7= 41.1, P < 0.001) (**Figure 4-2a**). Indeed, 5 studies did not find a significant difference in COMP level between early OA and control groups, while Wakitani et al. and Jiao et al. observed significantly increased COMP in early OA group and on the contrary Van Spil et al. found early OA group had lower serum COMP level.

Serum HA was assessed in 4 studies. One was longitudinal, and all studies included both males and females. Similar to COMP, the studies reported variable findings. Wakitani et al. and Jiao et al. found elevated HA in early OA while Van Spil et al. and Cibere et al. did not observe any significant difference between early OA vs. control groups. The overall SMD for HA was 0.15 (95% CI -0.101 to 0.402). Between-study heterogeneity was found among studies (I^2 = 75.83 %, χ^2_4= 16.551, P = 0.002) (**Figure 4-2b**).

NTX-I was evaluated in serum by Chaganti *et al.* and Kelman et al. and in urine by Van spil *et al.* and Cibere et al. One study was cross-sectional. Two studies were conducted on female subjects only. Kelman et al. reported a significant increased NTX-I in early OA group compared to control. Although the other studies observed a similar increment in NTX-I level in early OA group, the results did not reach significance. The overall SMD was small 0.066 (95% CI -0.015 to 0.147), with no significant heterogeneity found among studies (I^2 = 0 %, χ^2_4= 1.695, P = 0.792) (**Figure 4-2c**).

Although no statistical analysis applied, all other biomarkers were plotted in the same forest plot for easier representation of how results vary across the range of studies (**Figure 4-3**). The meta-regression analysis was applied on all studies and detected an

association between the SMD vs. sample size and affect joint (**Table 4-2**).

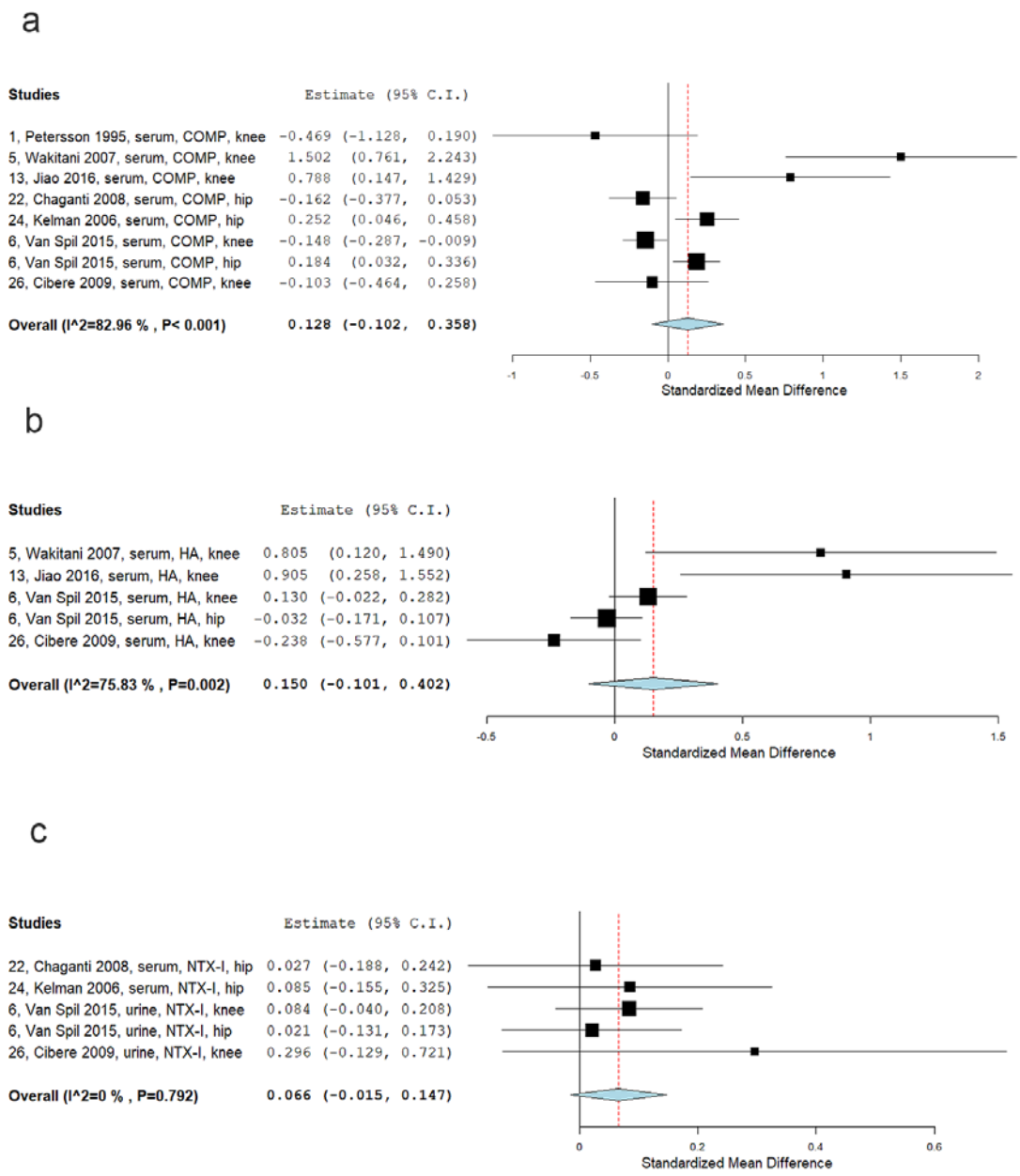


Figure 4-2. Forest plot of the effect size of COMP

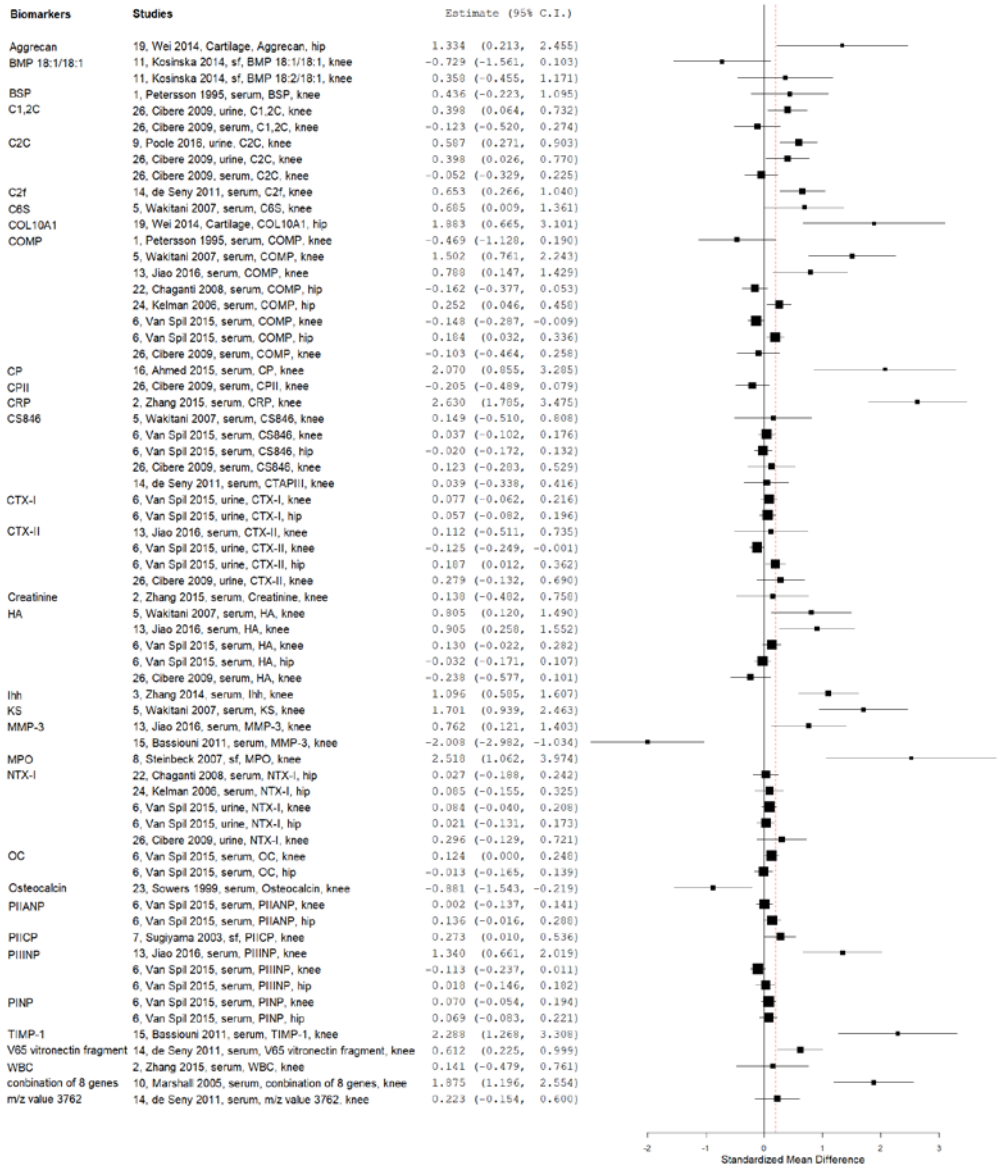


Figure 4-3. Forest plot of biomarkers

Table 4-2. Meta-regression of covariances related to heterogeneity

	Regression coefficient	p value	95% CI lower	95% CI upper
Age difference, early OA vs. control	-0.0145	0.2774	-0.0405	0.0116
Sex (female %)	-0.3817	0.3329	-1.1543	0.3909
Sample size	-0.0012	0.0083	-0.0021	-0.0003
Affect joint	-0.283	0.0561	-0.5734	0.0074

4.5 Discussion

In this review we have evaluated 35 biomarkers examined in 26 studies to assess if any specific biochemical marker was differentially expressed in early OA vs. healthy individuals. Three biomarkers, COMP, HA, and NTX-I were then synthesized using a meta-analysis of seventeen studies. Below, we will discuss each of these markers, and all present new opportunities for marker discovery and analysis that may aid in the development of efficient markers for the detection of early OA in the future.

Cartilage oligomeric matrix protein (COMP) is non-collagenous extracellular matrix (ECM) glycoprotein which contains thrombospondin-like domains that can bind to different collagen types (30) and is responsible for collagen–collagen interactions and microfibril formation in the cartilage. It is believed that COMP is primarily produced by articular chondrocytes and its elevation in bodily fluids is related to cartilage damage and/or turnover. Serum COMP concentration has been found elevated in multiple OA cohorts throughout many studies and is correlated with the severity of OA (30-33). Recent evidence has demonstrated that an elevated concentration of COMP can be detected in the fluid of injured joints years after the injury (34) or in the serum of OA patients after physical exercise (35) which indicates that COMP is a very sensitive

biomarker for asymptomatic early OA. However, despite all these studies, our review suggests that COMP levels in serum may not have a reliable diagnosis value for early OA. One reason behind this could be that subjects with early OA had not yet developed significant cartilage damage, therefore, the COMP level in the serum may dominated by the turnover of other types of cartilage in the body (e.g. costal cartilage) rather than the damage (if any) of the articular cartilage in the early OA joint.

HA, also known as hyaluronan, is a major component of synovial fluid and the extracellular matrix of the cartilage. It is believed that large molecular weight HA is responsible for lubricating the joint. However, in post-trauma or OA joints, the average molecular weight and the viscosity of synovial fluid HA decreases significantly comparing to normal individuals (49). The small molecular weight fragmented HA stimulates inflammation by binding to a number of cell surface PPRs (such as TLR4 and TLR2 as well as CD44) on various cell types including both leukocytes and non-leukocytes (such as fibroblasts and mesenchymal stem cells). HA have been found elevated in many OA cohorts (50-53). However, similar to COMP, a significant heterogeneity was observed between studies that may have contributed to the lack of efficacy observed in this review. Therefore, highly controlled studies with large sample sizes may be required in the future to validate the diagnostic role of HA for early OA.

N-terminal telopeptide of collagen type I (NTX-I) is another commonly investigated advanced OA biomarker. NTX-I is derived from the N-telopeptide portion of collagen type I. Since collagen type I is not the major structural component of articular cartilage (36), the level of NTX-I is unlikely to be a direct indicator for cartilage damage. It is

believed that subchondral bone turnover in the joints would affect the OA changes in some patients (147). In a prospective study, Doré et al. the authors found that subchondral bone changes may lead to cartilage damage. However, theoretically, It is difficult to use only NTX-I as an OA biomarker because systemic bone metabolism greatly affects its level (227). With that being said, we found that NTX-I was the only biomarker found to be significantly increased in early OA group by meta-analysis. This suggested that a complex relationship might exist between cartilage degeneration and bone turnover.

While we were only able to identify enough studies to examine these 3 biomarkers by meta-analysis, there have been an increasing number of biomarker studies in OA and early OA as the definition and understanding of the disease have been shaped in the last decade. Specifically, there is increasing evidence that OA is a chronic low-grade inflammatory disease rather than merely a “wear and tear” disease of synovial joints (20). A new definition of OA has recently proposed by The Osteoarthritis Research Society International (OARSI), emphasizing the role of inflammatory processes at the very early stage of OA:

“Osteoarthritis is a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by

cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness" (228).

Therefore, inflammatory biomarkers could be ideal candidates for asymptomatic early OA as they may directly or indirectly participate in the inflammatory processes that regulate the onset and/or progression of the disease. In fact, at the earliest stage of OA, it might be more feasible to detect inflammatory biomarkers rather than traditional cartilage metabolisms biomarkers which would be downstream of the inflammatory cascade. For example, in a cohort with traumatic meniscal injury but no radiographic evidence of OA, Scanzello et al. found that synovial inflammation was present in 43% of the patients and associated with pain and function scores (229). In another study conducted by Benito et al., synovial tissue from early OA (with normal radiographs) demonstrated increased inflammatory features than specimens from late stage OA patients, indicating that inflammation could activate pathophysiological pathways that contribute to downstream cartilage degradation. One main drawback of employing inflammatory mediators as biomarkers in OA is that many of the cytokines/chemokines/growth factors have little specificity for OA and/or early OA. Systemic inflammation can be regulated by a number of environmental and genetic confounding factors such as routine activity, obesity or even secondhand smoke exposure (230-232), which are difficult to control in cohort studies. In this review, we only found one study (220) investigating inflammatory biomarkers (IL-6, TNFα and CRP) in early OA, and disappointedly, its data format was not compatible for meta-

analysis. In the future, studies conducted thorough investigations of a variety of inflammatory markers, with highly controlled human cohorts or animal models could benefit the discovery of the biomarkers in early OA.

While the complexity of immune activation and inflammation in the pathogenesis of osteoarthritis is becoming increasingly well recognized, many biomarkers play redundant roles and involved in more than one signaling pathways which are not all related to the pathogenesis of OA. Moreover, it is difficult if not impossible to find a common molecular player in the multifactorial pathogenesis of early OA, including aging, injury, genetic predisposition and etc. Therefore, traditional studies focusing on quantitation of only one or a few biomarkers per experiment for correlation with a disease state is neither efficient nor effective for multifactorial diseases like OA. High-throughput “omic” technologies have had a revolutionary impact on early diagnosis of complex diseases as it facilitates broad scope of screening biomarkers (233). In this review, 3 studies utilized a set of biomarkers identified through "omics" techniques to differentiate early OA and control (210, 216, 218). Marshall et al. achieved 77% accuracy for classifying early OA vs control using a logistic regression model which combined the expression of 8 target genes identified from microarray analysis. Ling et al. detected 16 proteins that were different between early OA and control using a rolling circle amplification assay which could examine 169 unique proteins in one run. They then employed a decision tree algorithm around these 16 proteins and successfully classified early OA vs. control with over 94% accuracy. Ahmed et al. developed a

diagnostic algorithm containing 3 biomarkers identified by LC-MS/MS. This algorithm identified early OA with 73% sensitivity and 87% specificity. These 3 studies are examples that speak to the increase in accuracy when classifying groups with multiple compared to single biomarkers. These results and others suggested that early OA diagnosis could be considered in a more comprehensive manner rather than the traditional one marker – one disease strategy.

In the diagnosis of early OA it is also important to consider genetic factors, which in some cases can be a strong determinant of the disease (234). Genome-wide association studies conducted over the last decade have identified many SNPs that are associated with OA. However, in this review, inherited genetic disorders were not considered as biomarkers for early OA as they exist regardless the stage of the disease. Different from genetic factors, epigenetic factors can be dynamic. DNA-methylation and non-coding RNA are the most studied epigenetic factors in OA research (234). In the circulation, miRNAs are relatively stable, therefore, they might be promising easy-access biomarkers for early OA. Li et al. identified seven miRNAs which differentiate early and late knee osteoarthritis (235). Unfortunately, the study was lacking healthy control and therefore was not included in our qualitative analysis. lncRNAs also play a role in the development of OA. Pearson et al. discovered without two lincRNAs (CILinc01 and CILinc02), the IL-1 stimulated secretion of proinflammatory cytokines were significantly enhanced. By comparing damaged and non-damaged human knee articular cartilage, Bonin et al. found hypermethylated protein-coding genes FOXP4 and

SHROOM1, which were linked to OA pathology (236). In this review, we found no studies directly looked at epigenetic biomarkers in early OA. One reason may be the cost prohibitive nature of these assays in for large cohort studies. Nevertheless, the epigenetics field is developing quickly, and as the sequencing price decreases, future studies will benefit from large scale cohort studies examining epi/transcriptome profiles in early OA.

In conclusion, in this systematic review, 35 biomarkers for early OA were evaluated. Significant heterogeneity was observed in studies for COMP and HA, however, NTX-I was identified to be significantly increased in early OA patients by meta-analysis. Except for COMP, HA, and NTX-I, estimations of the SMD for the most biomarkers was limited by the small number of included studies, preventing an adequate statistical power. Large prospective and adequately powered studies are needed to validate the role of these and other biomarkers for identifying early OA.

Chapter Five: CCL22 is a biomarker of cartilage injury and plays a functional role in chondrocyte apoptosis

5.1 Abstract

Background: Knee osteoarthritis (OA) is one of the leading causes of disability worldwide. Previous history of knee injury is a significant risk factor for OA. Recently, it has been established that low-level chronic inflammation plays a pivotal role in the onset and pathogenesis of OA. The primary aim of this research was to determine if a history of knee joint injury is associated with systemic inflammation. A secondary aim was to determine if systemic inflammation is related to knee pain and joint structure.

Methods: Differences in serum cytokine association networks, knee joint structural changes (MRI), and self-reported pain (i.e., Knee Injury and Osteoarthritis Outcome Score Pain subscale, $KOOS_{PAIN}$ and Intermittent and Constant Osteoarthritis Pain score, ICOAP) between individuals who had sustained a youth (aged 15-26 years) sport-related knee injury 3-10 years previously and age- and sex-matched controls were examined. Proteins of interest were also examined in an OA rat model.

Results: Cytokine association networks were found to differ significantly between study groups, yet no significant associations were found with $KOOS_{PAIN}$ or MRI-defined OA. A group of cytokines (MCP1/CCL2, CCL22 and TNFα) were differentially associated with other cytokines between study groups. In a pre-clinical rat OA model, serum CCL22 levels were associated with pain ($r=0.255$, $p=0.045$) and structural changes to the cartilage. CCL22 expression was also observed in human OA cartilage

and furthermore, CCL22 induced apoptosis of isolated human chondrocytes.

Discussion: These results suggest that CCL22 may be an early factor in the onset/pathogenic process of cartilage degeneration and/or related to pain OA.

5.2 Introduction

Knee osteoarthritis (OA) is one of the leading causes of disability worldwide and is universally recognized as a major public health concern (197). There is increasing evidence that previous history of knee injury is a significant contributor to OA. Prospective studies have reported that knee injury increases the risk of developing radiographic knee OA by ~10 times (11) and it is estimated that 12% of cases of symptomatic OA in the hip, knee, and ankle are due to post-traumatic OA (PTOA) (237). In fact, more than 50% of individuals with an anterior cruciate ligament (ACL) tear or meniscus injury go on to develop PTOA (12, 13). Furthermore, meta-analysis indicated that even after ACL reconstructive surgery, there is still a 3.62-fold risk (range, 2.40-5.47; $P < .00001$) of developing PTOA 10 years after such injury (238). While this information speaks to the increased risk of developing PTOA following knee injury, the time from injury to OA can vary dramatically from years to decades (66). OA can only be clinically diagnosed at later stages when symptoms (e.g., pain, joint immobility) appear and joint damage/structural changes are severe enough to be detectable radiographically (3). Because of the lack of effective disease modifying treatments, knee arthroplasty aimed at restoring mobility and quality of life is often the final step

for patients with severe OA. Therefore, many researchers in the OA field, strongly believe that OA should be treated/managed at the earliest stages of the disease, when no symptoms have yet appeared and before radiographic evidence of OA is present (4, 239). While this would be ideal, we are yet unable to definitively identify patients at these early stages of the disease, and therefore novel methods for identifying early-stage OA are still required.

Early joint structural changes often include minor soft tissue damage, cartilage defects, meniscal damage, and bone marrow lesions (BMLs); most of these changes cannot be seen on radiographs and are observed using magnetic resonance imaging (MRI) (240). Sharma *et.al* reported that these MRI lesions could be representative of early OA as they were associated with the incidence of radiographic OA and subsequent persistent symptoms in participants who initially had Kellgren and Lawrence (K/L) = 0 (241). A similar study followed 50 individuals with knee scores of K/L = 0 that developed radiographic OA (K/L >= 2) 4 years later and found that cartilage T2 values at MRI could predict the development of radiological tibiofemoral OA (242). However, MRI identified minor tissue damage is also common in the general aged population (243), limiting the specificity of MRI-based prediction of future development of OA.

Intermittent, activity related pain is another sign of early OA (244). Pain is the hallmark symptom in late stage OA and is the major cause of disability in OA patients (197). Evidence has shown that there is a poor association between pain and radiographic or MRI findings (245), suggesting that pain is the result of a more complex mechanism than simply early state structural changes. However, animal studies have suggested that

OA pain is strongly associated with peripheral and central sensitization throughout the continuum of disease, and this process involves a variety of chemical mediators including inflammatory cytokines (246).

Based on pre-clinical and clinical research over the past decade, OA is no longer considered as simply “wear and tear”. Increasing evidence suggests that low-level chronic inflammation plays a pivotal role in the onset and pathogenesis of OA (247). Therefore, changes within the expression or activity of effector molecules (e.g. cytokines, growth factors, enzymes) could be prognostic before MRI detectable tissue damage or pain symptoms appear. Disappointingly, so far, no single biochemical marker has proven to be sufficiently robust for the diagnosis of early OA. One possible reason is the complex nature of the pathogenesis of OA, such that a single biochemical marker could be invalidated by various confounding variables including diet, physical activity, and systemic metabolism (19). To counteract these issues, high-throughput (omic) techniques with advanced, system-level analysis approaches have been employed for the development of OA diagnostics at the advanced/late stage by our group and others. However, these omic based approaches typically require using a broad range investigation of numerous factors (80). While not yet directly targeted to early OA, such assays consisting of broad-spectrum biomarkers combined with imaging and other techniques could be valuable for asymptomatic early stage OA.

Therefore, the purpose of this research project is to assess the association between systemic cytokines and knee injury history as well as clinical outcome measures indicative of early PTOA (BMI, self-reported pain and MRI-defined OA based upon

MOAKS scoring) in a youth knee injury cohort and validate these findings in a pre-clinical rat model of surgical induced OA.

5.3 Materials and Methods

5.3.1 Human Participants

This study included a sub-sample of 145 youth/young adults (15-26 years) from the Alberta Youth Prevention of Early OA (Alberta PrE-OA) cohort for whom baseline serum cytokine and clinical MRI were completed. Specifically, participants included 76 individuals who suffered a youth (under the age of 18 years) sport-related intra-articular knee injury 3-10 year previously and age, sex and sport (at the time of injury) matched uninjured controls. A description of the Alberta PrE-OA cohort, definition of the intra-articular knee injury, and recruitment and injury diagnoses procedures have previously been reported (66). Briefly, sport-related intra-articular knee injury was defined as a clinical diagnosis of knee ligament, meniscal or other intra-articular tibiofemoral or patellofemoral injury that required both medical consultation and disrupted regular sport participation. Injury diagnoses were based upon diagnostic codes recorded on previous cohort study injury report forms or University Sport Medicine Centre medical records or physician records and confirmed by participants. Uninjured participants reported no previous time-loss knee injury (6). Exclusion criteria included pregnancy, non-steroidal anti-inflammatory use or cortisone injection within three months prior to testing, a musculoskeletal injury within the previous three months prior to testing that resulted in time loss (i.e., work, school or sport), other arthritides,

or any current medical problem that prevented participation in the functional testing aspect of the study (e.g., neurological conditions). Ethics approval was granted from the Conjoint Health Research Ethics Board at the University of Calgary (REB14-2212), Canada and all participants provide signed consent/assent and completed a Physical Activity Readiness Questionnaire (PAR-Q, 2002) prior to testing. All testing was carried out in accordance with the declaration of Helsinki.

5.3.2 Procedures

After completing a custom designed study questionnaire (i.e., demographics, knee injury, surgery, medical history) the Knee Injury and Osteoarthritis Outcome Score (KOOS) and Intermittent and Constant Osteoarthritis Pain score (ICOAP), participants had their height (cm) and weight (kg) measured before serum samples were collected by a certified phlebotomist using standard venipuncture, with vacuum, non-treated tubes (66, 67). Serum samples were immediately aliquoted into pyrogen/endotoxin-free polypropylene tube and stored at -80^{0}C until required for analysis. All samples were only thawed once (at the time of analysis). Participants were recruited months later (147 days on average) for MRI studies at an offsite facility and reviewed by a musculoskeletal radiologist (JJ) with 13-years of imaging experience, blinded to injury history and intervention, using the MRI OA Knee Score (MOAKS). MRI defined-OA derived from MOAKS scores was based on established criteria (248, 249).

5.3.3 Body Mass Index

Body mass index (kg/m^2; BMI) was calculated from measurements of participants' height (to the nearest 0.1 cm; shoes removed) and weight (to the nearest 0.1 kg) assessed using a medical scale and stadiometer (Model 402KL, Pelstar, USA).

5.3.4 The Knee Injury and Osteoarthritis Outcome Score

The KOOS is a self-reported measure designed to evaluate knee related symptoms and function in young active patients with knee injury and OA. It has been validated in different populations varying in age, disease duration and activity levels and it has been shown to have high test-retest reliability (250). The KOOS consists of 42 items in five subscales: pain ($KOOS_{PAIN}$), other symptoms ($KOOS_{SYMPTOMS}$), function in daily-living ($KOOS_{ADL}$), function in sport and recreation ($KOOS_{SREC}$), and knee-related quality-of-life ($KOOS_{QOL}$)(251). Each item was scored on a 5-point Likert scale ranging from 'no problems' to 'extreme problems'. Subscale scores were then summed, and the total sub-scale score transformed to a 0-100 scale (higher scores indicating better outcome). For these analyses, only the scores from the $KOOS_{PAIN}$ subscale was considered.

5.3.5 Intermittent and Constant Osteoarthritis Pain Questionnaire

The Intermittent and Constant OA Pain Questionnaire is a self-report measure designed to evaluate constant and intermittent pain intensity including frequency and impact on mood, sleep and quality-of-life in persons with hip and knee OA (252, 253). As many

patients with knee OA report that their initial presentation of pain was intermittent, often during tasks such as climbing the stairs (254), the ICOAP, which includes a subscale for intermittent symptoms was seen as particularly relevant to the cohort under investigation. This self-report questionnaire consists of 11 items forming two subscales (5 items addressing constant pain and 6 items addressing intermittent pain). The ICOAP has good internal consistency, test retest reliability (ICC=0.85) and construct validity when compared to KOOS and Western Ontario and McMaster Universities Arthritis Index Scores (252), Each item was scored on a 5-point Likert scale ranging from no pain to high (disability-severely limiting) pain. Sub-scale scores were then summed, and the total sub-scale score transformed to a 1-100 scale (higher scores indicating poorer outcome).

5.3.6 Magnetic Resonance Imaging

Participants underwent bilateral knee MRI studies using typical clinical projections and sequences. Sequences included: Sagittal proton density (PD) TR/TE 1500/10 ms, slice thickness 3.5 mm, field of view (FOV) 150x140 mm; Sagittal and coronal PD fat saturated (FS) with TR/TE 2660/28 ms slice thickness 3.5 mm, field of view (FOV) 150x140 mm; and 3D gradient echo FIESTA sequence with TR/TE 10.5/4.2 ms, flip angle 55°, slice thickness 1.0 mm and isotropic voxels. All studies were rated by a musculoskeletal fellowship trained radiologist (JJ) with 13-years of imaging experience, blinded to injury history or surgical intervention using the semi-qualitative MRI OA Knee Score (MOAKS) (248, 255). MRI defined-OA derived from MOAKS scores was

based on established criteria (248). The intra and inter-rater reliability of MOAKS scoring for this sample has been previously reported (kappa=0.61–1.0) (67).

5.3.7 Multiplexed Arrays (Human Samples)

Sample analysis was performed by Eve Technologies (Calgary, AB Canada) using the Milliplex MAP Human Cytokine/Chemokine Panel (Millipore), according to the manufacturer's instructions. All samples were assayed at least in duplicate and prepared standards were included in all runs. The following proteins were examined by Luminex in this study for human serum samples: EGF, Eotaxin, FGF2, Flt3L, Fractalkine, GCSF, GMCSF, GROα, IFNα2, IFNγ, IL1α, IL1β, IL1rα, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12 (p40), IL12 (p70), IL13, IL15, IL17A, IL18, IP10, MCP1, MCP3, CCL22, MIP1α, MIP1β, PDGFAA, PDGFAB/BB, RANTES, sCD40L, TGFα, TNFα, TNFβ, VEGFA. The sensitivities of these makers range from 0.1 – 10.1 pg/mL (average 2.359 pg/ml) and the inter-array accuracies range from 3.5% – 18.9% coefficient of variation (average 10.7%).

5.3.8 Animal model

Sham controls and standardized joint injuries (destabilization of the medial meniscus; DMM) (256) were induced on the left knee joints of rats (n=18). Three rats were sacrificed at one, two and three, weeks after injury, and nine rats sacrificed at the fourth week. To minimize the individual variance, only the nine rats sacrificed at the 4th week were used for repeat pain and serum cytokine profile studies starting before the injury

(day 0). Specifically, before injury and 3, 5, 10, 14, 20, 24 days after injury, serum samples were harvested and processed as described above for cytokine profile analysis.

5.3.9 Multiplexed Arrays (Animal Model)

Milliplex MAP Rat Cytokine/Chemokine Array 23-plex was used and the following cytokines were examined: EPO, IL13, IL10, IL18, IL1α, IL2, MCSF, IL1β, IL4, IFNγ, MIP3α, GMCSF, IL7, TNFα, VEGF, MCP1, IL5, GCSF, RANTES, IL6, GRO, IL17α, IL12p70. Unfortunately, a rat cytokine array matched with the human cytokine array was not available. Therefore, the concentration of CCL22 in rat serum was analyzed independently using sandwich ELISA (LS BIO) following the manufactures instructions. Missing data were imputed using regression imputation from RANTES in the Miliplex array (β=-0.684, constant=4511.337, $p<0.001$).

5.3.10 Rat Grimace Scale (RGS)

Application of the RGS was based on scoring randomised, blinded images of individual rats (257, 258). Briefly, each rat was video-recorded for 15 minutes at each time point in a plexiglass video chamber (W 14 cm x L 26.5 cm x H 20.5 cm). Recorded video was reviewed by a trained observer (blinded to treatment and time point) and an image captured every 3 minutes. No images were collected during the first minute of recording to allow the rat to acclimate. Image selection criteria were: absence of movement artifact, a clear view of all relevant facial features (nose, cheek, eyes, ears) and absence of directed behaviours (grooming, rearing, sleeping). Generation of a score with the

RGS requires assessment of four “action units”: orbital tightening, nose/cheek flattening, ear changes, and whisker change. Each action unit was assigned a score of 0, 1 or 2, and the four scores averaged to generate a single RGS score for each image. A score of “0” reflects an absent action unit, a score of “1” indicates the moderate appearance of an action unit, and a score of “2” indicates the obvious appearance of an action unit (associated with a painful state).

5.3.11 Histology and Immunofluorescent (IF)

Both injured and uninjured (control) rat joints and human cartilage samples were fixed with formalin and embedded in paraffin. Intact knee joints were dissected and fixed in 4% normal buffered formalin (Sigma, St. Louis, MO). Samples were decalcified and embedded in paraffin (VWR, Radnor, PA). Ten μm thick, longitudinal serial sections were stained with Safranin O (Fisher, Waltham, MA) to visualize proteoglycans. In the rat, whole joint sections, medial, lateral and the ACL/PCL insertion sites were graded for signs of OA according to the OARSI Guidelines for rat knee joints (259). Sections were deparaffinized in CitraSolv (Fisher Scientific; Fairlawn, NJ) and rehydrated through a series of graded ethanol to distilled water steps. Antigen retrieval (10mM sodium citrate, pH 6.0, brand) and blocking (1:500 dilution; 100μL rat serum (or goat serum for human samples): 50mL TRIS-buffered saline, 0.1% Tween 20 (TBST) for 1hr), steps were performed prior to going through sequential wash (TBST) and primary antibody application steps. Primary antibodies directly conjugated to fluorescent probes (Abcam, dylight system) for CCL22 (rat: Cat. No. bs-1761R, Bioss) (human: Cat. No.

MAB336, R&D systems); cleaved caspase 3 (rat & human: Cat. No. 9661, Cell Signaling Technology) and the nucleic acid stain DAPI (Sigma) were applied to sections. After antibody staining, sections were mounted using FluorSave reagent (Calbiochem) and coverslipped. A Zeiss Axio Scan.Z1 microscope was used to detect the signal for each antibody.

5.3.12 Human Cartilage Biopsies

OA Cartilage biopsies were collected from knee OA patients (n = 8; 3M/5F, mean age 55.5 ± 7.1 years) undergoing joint replacement. Samples were processes using methods outlined in Histology section. Normal cartilage samples (n=3; 2M/1F, mean age 54.2 ± 5.3 years) were obtained from the Southern Alberta Tissue Donation Program. Criteria for control cadaveric donations were an age of 40 years or older, no history of arthritis, joint injury or surgery (including visual inspection of the cartilage surfaces during recovery), no prescription anti-inflammatory medications, no co-morbidities (such as diabetes/cancer), and availability within 4 hours of death. Samples were processes using methods outlined in Chondrocyte Isolation and Culture section.

5.3.13 Chondrocyte Isolation and Culture

Cartilage tissue was cut in pieces of approx. $2mm^2$, then incubated with 1mg/ml pronase (Roche, Cat. No. 1459643) for 30 minutes at 37°C (100 rpm). The cartilage was then incubated with 1mg/ml collagenase (Serva, Cat. No. 17465) for 24 hours at 37°C (100 rpm). The resultant suspension was filtered (70μm) and centrifuged. The chondrocytes

were re-suspended in DMEM/F-12 (Gibco Cat. No. 31330) supplemented with 10% FBS and Anti-Anti. Chondrocytes were incubated with recombinant human CCL22 (Peprotech) or PBS alone and analyzed for Annexin V staining (Thermo Cat. No. BMS500FI) using flow cytometry. Chondrocytes were also stained for CCR4 (Cat. No. 557863, BD Biosciences).

5.3.14 Data Analyses

Statistical analyses were performed using SPSS and R. The participants with missing values were removed from the analyses. The differences of sex, age, BMI, and MRI-defined OA between injured and uninjured group were evaluated using student's t test (α=0.05). Univariate logistic regression was used to ascertain the effects of age, sex, BMI, $KOOS_{PAIN}$ and $ICOAP_{TOTAL}$ pain on the likelihood that participants had a previous knee injury. The between-group differences for individual cytokines were accessed by Student's t test. The correlations between cytokines and pain scores were determined using Spearman's rank test. Multiple comparisons correction was not applied in this study as experiments were implemented to validate statistic findings.

Cytokine association networks from cytokine profiles of participants with or without a history of injury were constructed independently using the ARACNe algorithm as followed (260). First, the pairwise mutual information (MI), which was considered as the strength of pairwise cytokine association were estimated for all cytokine pairs. Secondly, by applying the data processing inequality, most indirect associations (lowest strength, or smallest MI value of any 3-cytokine loops) were removed. Finally,

networks were constructed with cytokines as nodes and remaining associations as weighted connecting lines. To quantify these perceived differences between networks, the change of centrality (betweenness) of each cytokine was computed. Betweenness has been widely applied in the analysis of biological networks (261) and other types of networks in general (262). In graph theory, the betweenness of a node measures the number of times it acts as a bridge along the shortest path between all node pairs in the same network. Therefore, in this study, the cytokines with high betweenness can be considered as key connectors within the network, and the change of betweenness of a cytokine between two networks can therefore be considered as an indicator of the topological change of this cytokine. Networks were visualized using Cytoscape 3 with DyNet package (263, 264).

Cytokine association networks of the rat at 7-time points (before and 3, 5, 10, 14, 20, 24 days after injury) were created in the same method described above. The connectivity (average node betweenness centrality) of each network of 7 time-points were calculated for the correlation analysis with rat pain score data.

5.4 Results

5.4.1 Participant Demographics

The demographics of the participants (n=145) are summarized in **Table 5-1**. The median age of participants was 23 years (range 15-27) and 88 were female. The median age of injury was 16 years (range 11-19) and the median time between injury and data/sample collection was 6.9 years (range 4-10). There were no significant differences

in sex, age between the injured and uninjured group (**Table 5-1**). The injured study group had lower BMI (p=0.003), lower $KOOS_{PAIN}$ (p<0.001) and more MRI-defined OA (p<0.001) than the uninjured group.

Table 5-1. Participant Characteristics, BMI, $KOOS_{PAIN}$, $ICOAP_{TOTAL}$ and MRI-defined OA by Study Group

Outcome		Injured (n=76)	Uninjured (n=69)	p-value	95% C.I.	
					Lower	Upper
Sex (%female)		45	43	0.896	0.579	1.869
Age (years; median, range)		23 (15– 27)	23 (18-27)	0.246	0.984	1.004
BMI (kg/m^2; median, range)		23.8 (18.6-31.3)	24.9 (19.4-38.9)	**0.003***	1.056	1.294
MRI defined OA (n)		44	19	**<0.001***	0.202	0.628
Pain	**$KOOS_{PAIN}$ (median, range)**	94 (58-100)	100 (69-100)	**<0.001***	0.808	0.939
	$ICOAP_{TOTAL}$ (median, range)	5 (0-43)	2 (0-36)	0.144	0.871	1.020

5.4.2 Comparing individual cytokines in cytokine association network for injured vs. uninjured participants

Cytokine association networks were created for both injured and uninjured groups based on the serum levels of 41 distinct analytes. It was found that most cytokines were visually different between two networks (injured vs. uninjured) in terms of their topological patterns (e.g. what "association partners" they connected to) (**Figure 5-1**). By comparing the betweenness of cytokines in two networks, MCP1/CCL2, CCL22

and TNFα demonstrated the greatest difference in network connectivity between the injured vs. uninjured cytokine networks (**Figure 5-1**).

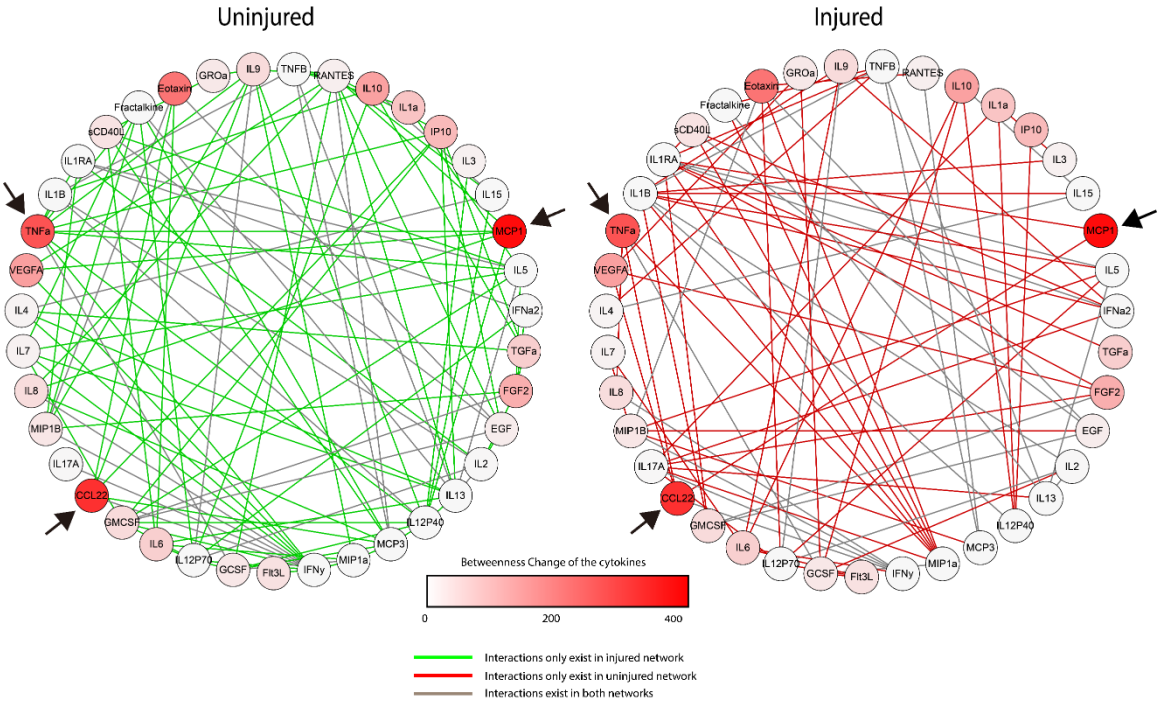


Figure 5-1. Networks comparison between injured and uninjured. The injured vs. uninjured cytokine networks were different in terms of the connectivity between cytokines. The connections that only existed in injured (green), uninjured (red) and both (grey) are presented. The absolute betweenness differences between injured vs. uninjured networks per cytokine were calculated and are represented by the shade of red color for each cytokine. The position of MCP1/CCL2, CCL22 and TNFα are marked by arrows.

5.4.3 Comparing overall difference between injured vs. uninjured networks

To statistically compare the overall difference between injured and uninjured networks, a permutation test was developed which involved a null hypothesis regarding the

average betweenness of cytokines in networks. The null hypothesis stated that if cytokine association networks N_1 and N_2 are randomly organized, then N_1 and N_2 should have the same connectivity (average betweenness of all cytokines). Formally, the null hypothesis H_0 is:

$$H_0: C_\delta = \overline{C_1} - \overline{C_2} = 0$$

Where $\bar{C_1}$ and $\overline{C_2}$ are the average betweenness centrality of all cytokines in network N_1 and N_2.

The distributions of C_δ were generated empirically through 100,000 permutations (randomly re-assigning group labels and repeated 100,000 times). The two-tailed p-value was computed as

$$p_{two-tailed} = \frac{N_{|C_{\delta_{obs}}| \leq C_{\delta_{sim}}}}{N_{perm}}$$

Where $N_{|C_{\delta_{obs}}| \leq C_{\delta_{sim}}}$ is the number of simulated C_δ that are larger than the absolute value of observed $C_{\delta_{sim}}$. N_{perm} is the number of permutations which is 100,000 in this study.

The connectivity was significantly higher in injured than uninjured network (p=0.006, **Figure 5-2**). On average, the injured network consisted of more "key cytokines" that demonstrated bridges/associations to the remainder of cytokines examined. This indicated a non-random or coordinated change in cytokine levels following injury, which suggested that cytokines in the injured group were more associated and co-regulated with each other.

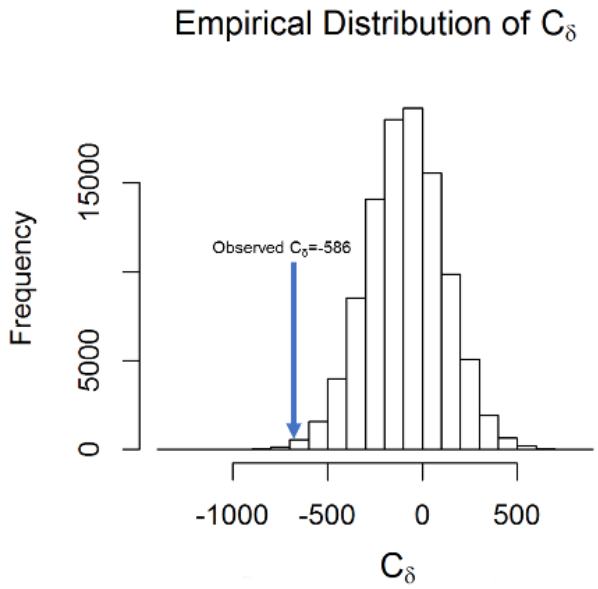


Figure 5-2. Permutation test of cytokine networks between the groups with or without a history of joint injury. The distributions were based on simulated data and N_{perm} = 100,000 permutations.

To test if the network results were distinct between females vs. males, the dataset was dichotomized by sex and the previous network analysis was run for both subsets separately. In the male subset, the 3 most differentially regulated cytokines were Eotaxin, IP10 and EGF. In the female subset, the 3 most differentially regulated cytokines were EGF, TGF and RANTES. The overall difference between injured vs. uninjured network was not significant in male (p= 0.381) or female (p= 0.379) subsets.

5.4.4 Cytokine profile comparison using classic statistic approach

While the network analysis was able to identify the difference between injured vs. uninjured cytokine networks as a whole and determine which nodes (individual cytokines) most differed between networks; this analysis was unable to determine if

any given cytokine demonstrated a difference in expression between injured vs. uninjured cohorts. Therefore, classical statistic approaches were applied to supplement the findings in the previous network analysis. Multivariate Analysis of Variance (MANOVA) was used to compare cytokine profiles between injured vs. uninjured cohorts. When all analytes were considered together, the two profiles were significantly different ($p = 0.001$). This was consistent with the analysis using cytokine association networks. However, no significant difference was found within any individual cytokines between two cohorts using Student's t test with Bonferroni correction. Moreover, since the injured group tended to demonstrate worse pain scores and MRI-OA scores than the uninjured cohort, the correlations between cytokines and pain and MRI-OA scores were tested, but none were found to be significant. Although not in direct disagreement with the previous finding, this strongly suggests that a factor other than cytokine concentration alone is responsible for the difference observed between networks. It is therefore very likely that cytokines association is playing a role in the network analysis and this is not accounted for with univariate methodologies.

5.4.5 Cytokines correlated with pain in rat OA

Among the most different cytokines, MCP1 and TNFα were found elevated in osteoarthritic joints in previous studies and shown to correlate with the initiation and progression of OA (265). However, very few studies have reported the correlation between CCL22 and OA. Since it was not possible to determine if CCL22 expression was directly related to the previous joint injury in the human cohort study, a pre-clinical

rat model was used to validate the cytokine association network analysis as well as the role of CCL22 in the initiation/early stages of OA. Standardized joint injuries (destabilization of the medial meniscus, DMM) were induced on the left knee joints of rats (N = 18). Half of the animals were used for repeated serum cytokine analysis, the remainder were sacrificed at different time points after injury for histology analysis of the joint and CCL22 expression/localization.

The expression of twenty-four cytokines/chemokines/growth factors were examined in the serum of rats (pre-injury vs. after DMM) and found to be similar to the analysis conducted in the human cohort study, the connectivity of rat cytokine association network for uninjured (before injury) and injured (days 3-24) were calculated. The network connectivity was increased after the joint injury, which was consistent with the result from the human cohort. Moreover, the connectivity of the network was positively correlated with rat pain ($r= 0.786$, $p = 0.036$) at the different time points examined after DMM injury (**Figure 5-3**). This indicated that in this rat model, the induction of the injury led to an increase in pain, with a corresponding increase in connectivity of in the cytokine association network. Furthermore, CCL22 was also found positively correlated with pain ($r=0.255$, $p=0.045$) after joint injury within the rat model while most other cytokines were negatively correlated with pain (**Supplementary Table 9-5**).

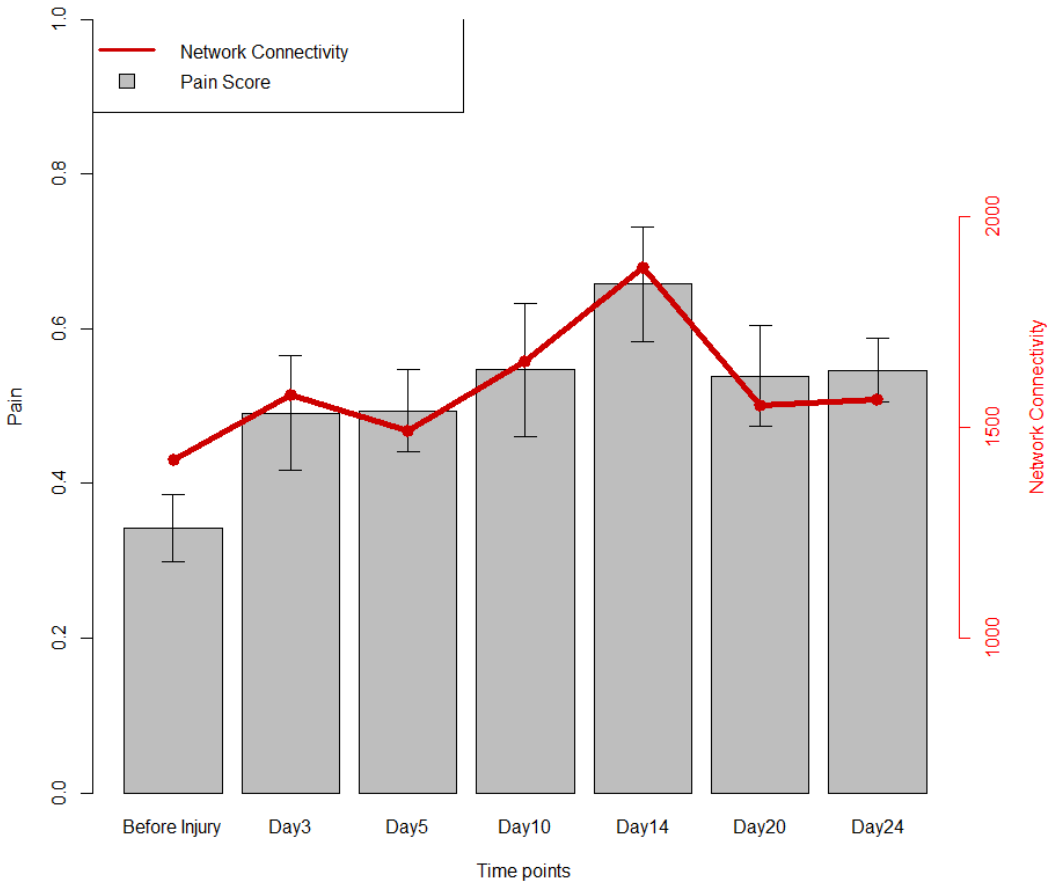


Figure 5-3. In a rat DMM model, cytokine associations are correlated with joint pain after injury. The average pain score of rats that underwent DMM surgery (n=9) at different time points after surgery is shown in the bar graph. The connectivity of the cytokine network for each time point are represented as the red line. The bar graph and line demonstrated a similar relationship at each of the time points examined, and they were found to be statistically correlated (r= 0.786, p = 0.036).

5.4.6 CCL22 association with structural changes within the rat joint

Since it was observed that CCL22 was also correlated with MRI structural changes within the human cohort, the correlation between OARSI OA histological scoring and serum CCL22 concentration on before the scarification (4 weeks post injury) were accessed in the rat DMM model. Immunofluorescence (IF) was employed to detect the

presence and localization of CCL22 in uninjured rat joints in additional to rat injured joints harvested at 1, 2, 3 and 4 weeks after the induction of injury (DMM). The OARSI OA scores and IF findings of all joints are shown in **Supplementary Table 9-6**. Serum CCL22 expression levels were correlated with cartilage degeneration width (r=0.905, p=0.002) and surface matrix loss (r=0.786, p=0.021) (**Supplementary Table 9-7**). Upon examination of joint tissue with IF, CCL22 was detected in the cartilage or/and synovium in all joints (n=12) with visible damage (total degeneration width > 0 um) (**Figure 5-4**). Within injured joints without cartilage degeneration, osteophytes or synovial inflammation, CCL22 staining was present 3 out of 6 joints and through comparing the histology and IF staining of adjacent slides in these joints without visible damage, CCL22 were found to be only present in the areas of cartilage where noticiable proteoglycan loss was observed (**Figure 5-4**).

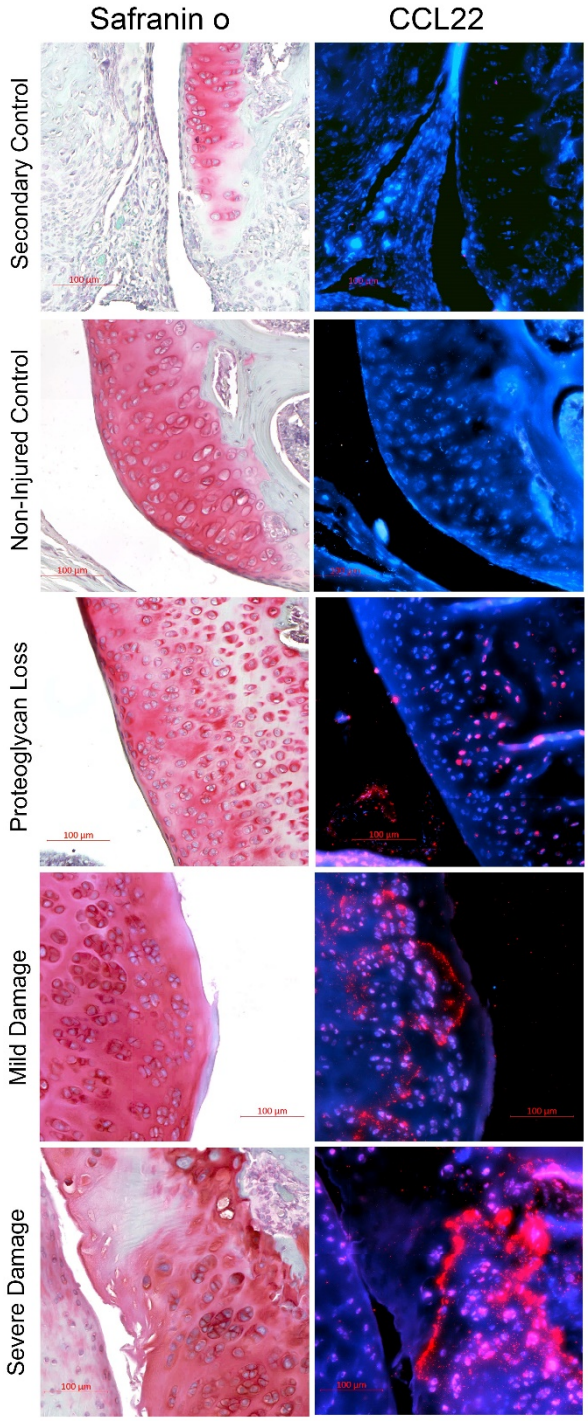


Figure 5-4. IF and histology staining of cartilage & synovium. IF and histology slices of serial sections are presented in each row. Safranin O stained sections depict proteoglycan staining (red), while IF staining demonstrates CCL22 staining (red) in relation to DAPI nuclear staining (blue). Scale bars = 100µm.

5.4.7 CCL22 association with chondrocyte apoptosis markers within rat cartilage

Since chondrocyte death is a hallmark of OA and it was observed that chondrocytes in areas of cartilage damage stained positive for CCL22 expression (**Figure 5-4**) it was examined if CCL22 was co-localizing with markers of chondrocyte apoptosis. Healthy rat cartilage and areas of cartilage demonstrating increasing levels of damage were doubled stained with CCL22 and the apoptosis marker cleaved caspase 3 (**Figure 5-5**). In healthy cartilage, little to no CCL22 and/or cleaved caspase 3 was detected, however as cartilage damage increased, an increase in CCL22 and cleaved caspase 3 positive cells were observed (**Figure 5-5**). Furthermore, a high degree of co-localization between CCL22 and cleaved caspase 3 was observed in damaged cartilage (**Figure 5-5**).

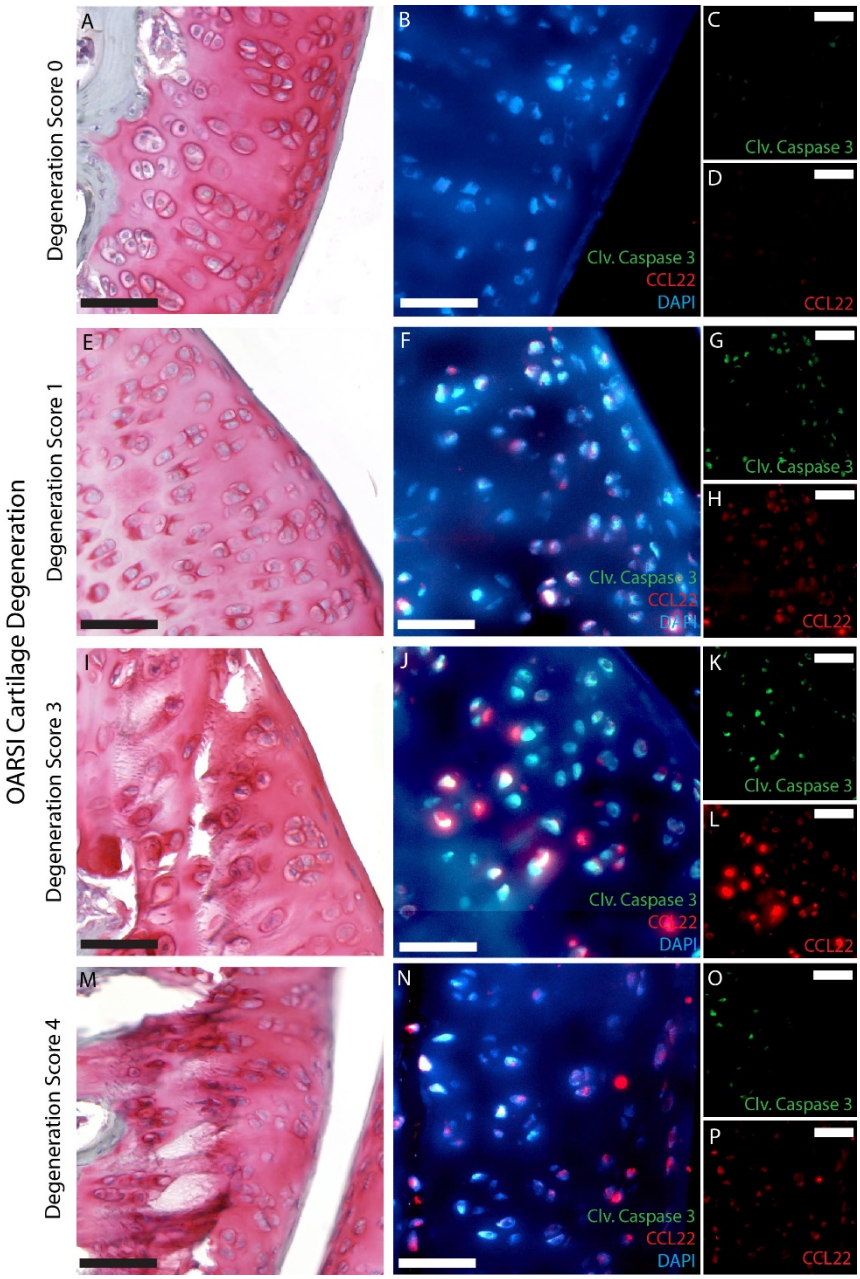


Figure 5-5. Co-localization of CCL22 and cleaved Caspase 3 in chondrocytes. IF and histology slices of serial sections are presented in each row. Areas of cartilage were classified by increasing (worsening) OARSI degeneration score based on Safranin O stained sections (A,E,I,M). Serial sections were stained with CCL22 (red) and cleaved (Clv.) caspase 3 (green) (B,F,J,N). As the OARSI degeneration score increased, so did CCL22 and cleaved (Clv.) caspase 3 positive chondrocytes in the cartilage. Furthermore, apoptotic chondrocytes (cleaved caspase 3 positive) also presented with positive CCL22 staining. However, not all CCL22 positive cells were also positive for cleaved caspase. Scale bars = 100µm.

5.4.8 CCL22 association with chondrocyte apoptosis within the human cartilage

To validate the expression of CCL22 and it co-localization within apoptotic chondrocytes in human cartilage, biopsies were obtained from patients undergoing joint arthroplasty and stained for CCL22 and cleaved caspase 3 (**Figure 5-6**). CCL22 and cleaved caspase 3 staining was observed in OA cartilage, while CCL22 expression was also observed in the subchondral bone and bone marrow. Furthermore, as observed in damaged rat cartilage, CCL22 positive staining was observed in chondrocytes undergoing apoptosis (e.g. cleaved caspase 3 positive) (**Figure 5-6**).

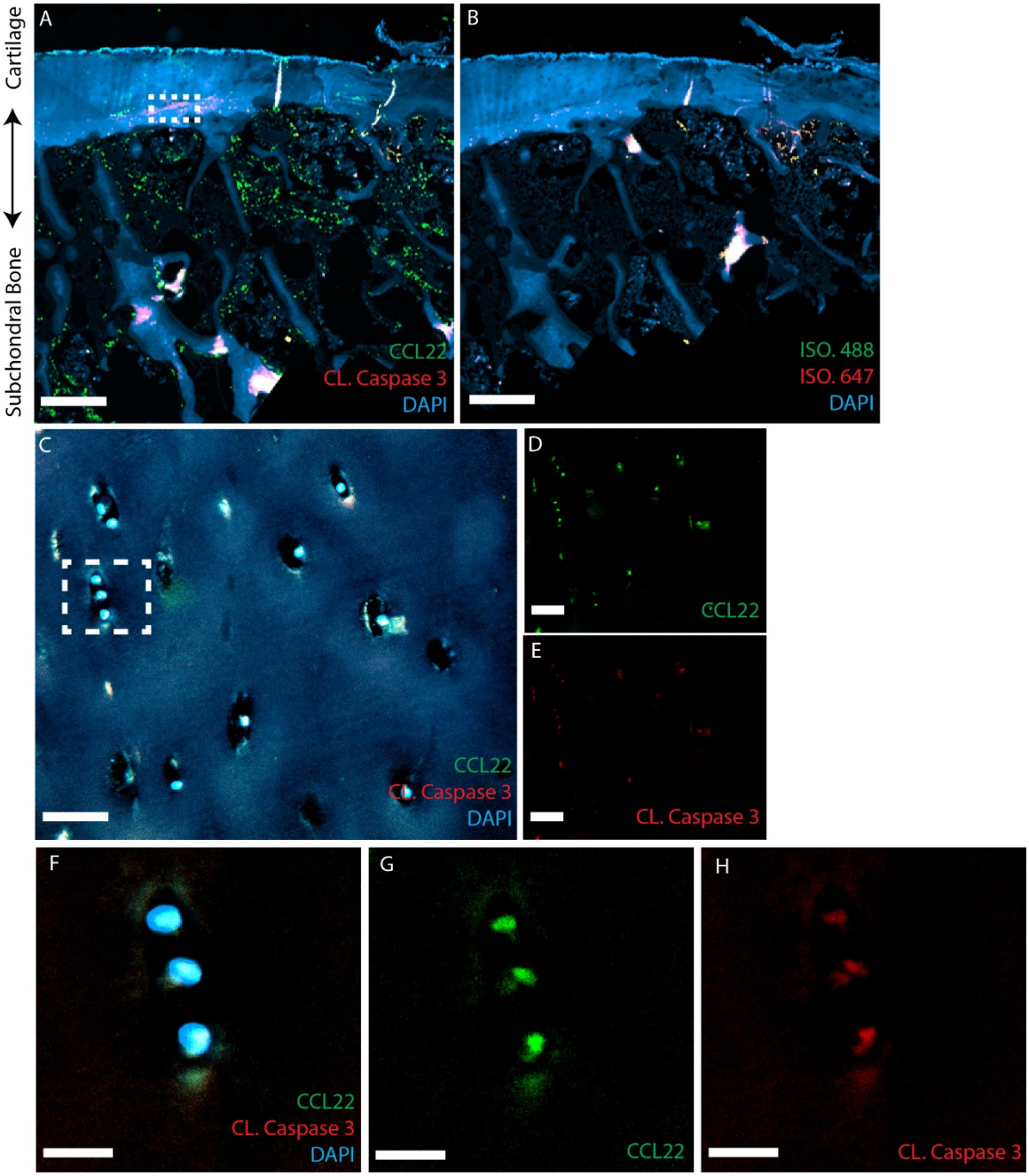


Figure 5-6. Co-localization of CCL22 and cleaved Caspase 3 in human cartilage/chondrocytes. Cartilage sections were stained with CCL22 (green) and cleaved (Clv.) caspase 3 (red) (A, C-H) or isotype controls (B). Chondrocytes within OA cartilage demonstrated expression of both CCL22 (C,D) and cleaved caspase 3 (C,E). Furthermore, apoptotic chondrocytes (cleaved caspase 3 positive) also presented with positive CCL22 staining (F-H). Scale bars for A,B = 200µm; for C-E = 50µm; for F-H = 25µm.

5.4.9 CCL22 induces chondrocyte apoptosis

To determine if CCL22 was playing a role in the apoptosis of chondrocytes compared to co-expression in areas of damaged/dying cartilage, human chondrocytes were isolated from cadaveric donors without OA/cartilage pathology and exposed to increasing concentrations of CCL22. Apoptosis was analyzed by Annexin V staining, and the cells were doubled labeled with CCR4, the only known receptor for CCL22. The concertation range of CCL22 employed was determined from previous studies on the concentration range of CCL22 in the synovial fluid of patients with OA (2953pg/ml +/- 3628pg/ml). The negative control (PBS) demonstrated minimal Annexin V staining (mean = 3.14%) while increasing concentrations of CCL22 increased in the percentage of apoptotic cells (**Figure 5-7**). The most profound effects were observed at 3ng/ml and 10ng/ml where 57.23% and 82.21% of chondrocytes stained positive for Annexin V respectively (**Figure 5-7**). It should also be noted that while approx. half of chondrocytes expressed CCR4 in the PBS treated cells, the number of CCR4 positive chondrocytes in the population increased with increasing concentrations of CCL22.

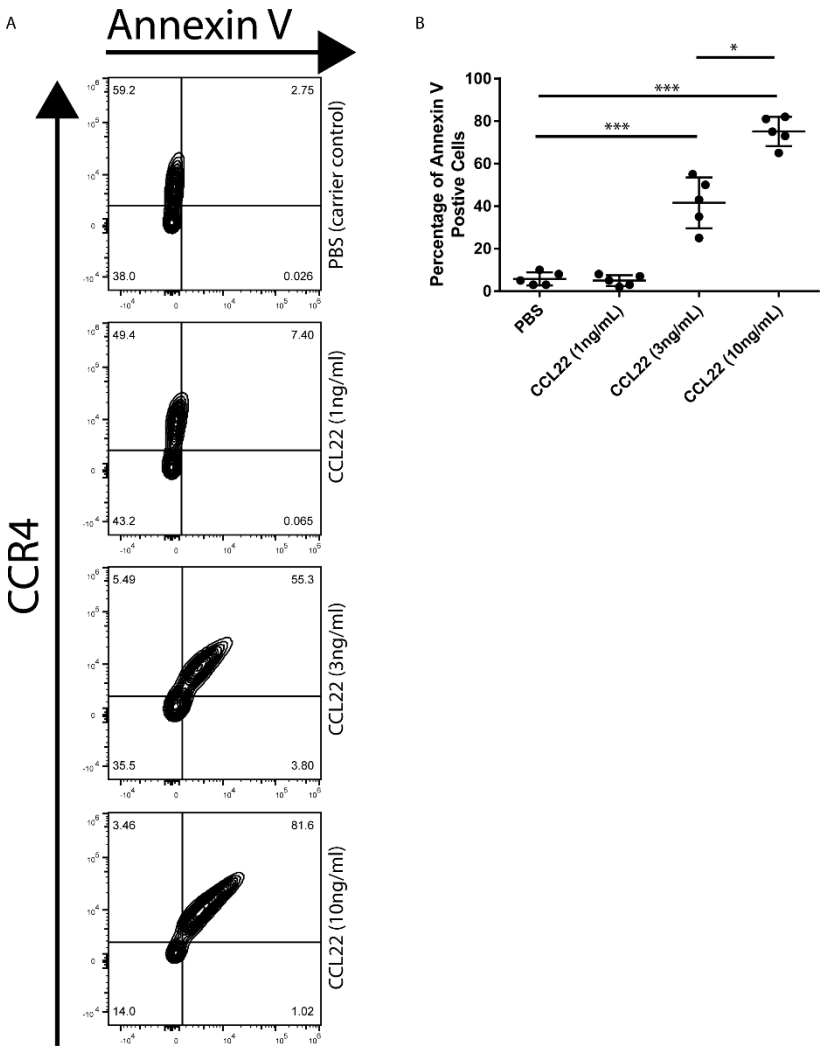


Figure 5-7. Apoptosis in isolated human chondrocytes exposed to CCL22. Chondrocytes were isolated from individuals without OA and assayed for apoptosis (Annexin V) and CCR4 (CCL22 receptor) with increasing exposure to CCL22. As CCL22 concentrations exceeded 3ng/ml, increased levels of Annexin V / CCR4 double positive chondrocytes were observed. Chondrocytes treated with PBS (carried control) alone and/or 1ng/ml of CCL22 demonstrated minimal Annexin V staining. Chondrocytes from 2 donors are presented.

5.5 Discussion

This study investigated cytokine networks based on the expression of 41 cytokines in individuals that had suffered a sport-related intra-articular knee injury 3-10 years previously in their youth compared to age- sex- and - sport-matched (sport at the time

of injury) uninjured controls. Although many of the cytokines have been studied individually in advanced OA cohorts (165), to our knowledge, this is the first study focusing on cytokine networks as a whole in a young cohort without a clinical diagnosis of OA.

Many studies have confirmed OA pain is not always consistent with radiographic or MRI structural changes (245, 266, 267). In fact, using the data from this cohort, we have previously reported that we found no evidence of a linear correlation between MRI–defined OA and knee symptoms or function (67). Although many cytokines have been reported to be responsible for OA pain (176), in this study, we have found no evidence of a correlation between cytokines and $KOOS_{PAIN}$ or $ICOAP_{TOTAL}$ scores. This might reflect that 1) most of the participants in the current study were pre-disease, 2) and/or regardless of disease state were generally asymptomatic, 3) it could be possible that any pain due to the previous injury and/or disease may also have been episodic, 4) and lastly, there may be no (or potentially a weak correlation between pain and structure. Cytokines are known to be pleiotropic and inter-dependent. As a result, when studying a specific cytokine, ignoring its dependency to/with other cytokines will result in the loss of important information, which could lead to an erroneous conclusion. For example, IL6 can be both anti- and pro-inflammatory through different signaling pathways at the same time (268). Therefore, it is reasonable to look at the relationships between IL6 and its downstream cytokine expressions rather than the level of IL6 alone, to determine which role IL6 is currently playing. In a recent study that analyzed the pairwise correlations between 17 cytokines, Wallner *et al.*, found that drugs modulated

cytokine correlations differently instead of causing a general inhibition of the cytokines (269). This was similar to our finding that injury altered the inflammatory responses that were reflected in overall cytokine associations rather than simply increasing/decreasing the absolute concentration of each cytokine. Because of the complexity (e.g. redundancy, routes of feedback and cross-talk) of cytokine signaling, it is neither appropriate, nor practical, to isolate any single cytokine and its associations from the complete network of cytokines interactions and study them individually. The analysis of biological networks has shown its advantage in understanding complex biological systems as a whole (270-272). However, even though comparing network structure was involved in most of the studies, very few had statistically evaluated the topological difference between networks. In this study, we developed a permutation test to evaluate the difference of connectivity between networks. The result was consistent with MANOVA applied on original multivariate datasets.

It is known that a history of injury is a significant contributor to knee OA, and chronic inflammation seems to play a pivotal role in the pathogenesis of OA (273). Does injury alter the local inflammatory environment leading the degeneration of the joint? To address this question, we compared cytokine profiles between participants with and without a history of injury. We found the two groups were significantly different when comparing the cytokine profile by computing the cytokine network connectivity or by using multivariate analysis. The network of injury group had a higher average betweenness, meaning that on average the cytokines of the previously injures participants had a stronger association with each other than seen in the uninjured

participants. This suggests that on average the cytokines of the previously injured participants are more highly co-regulated than in the uninjured participants. Although none of the individual cytokines were significantly different when comparing the means between injured vs. uninjured, most cytokines were generally different between the two groups in terms of their "connectivities" in the cytokine association networks. Among the most different cytokines, MCP1 and TNFα were found elevated in osteoarthritic joints by a variety of studies previously and have been shown to be correlated with the initiation and progression of OA (176, 274). But very few studies have reported a correlation between CCL22 and OA. CCL22 has a complex role in inflammation as it is chemokine for both Th2 and Treg cells (275, 276). It has been reported that CCL22 and its receptor CCR4 were found in the synovial membrane of osteoarthritis patients (277) and expressed by subpopulations of sensory neurons (278). In the rat model of this study, CCL22 was found correlated with pain. Moreover, CCL22 was present not only in severely damaged joints in rats but also in the cartilage before the visible damage occurred. Furthermore, CCL22 expression was observed in chondrocytes undergoing apoptosis (cleaved caspase 3 positive cells), with almost all apoptotic cells being positive for CCL22. This result was validated in human OA cartilage, and we were also able to demonstrate a functional role of CCL22 in the induction of apoptosis of human chondrocytes. While chondrocyte apoptosis is a hallmark of OA (279), to our knowledge CCL22 has not been previously implicated in the induction of apoptosis of chondrocytes and/or any other cell type. However, it has been previously demonstrated the TNFα plays a role in chondrocyte apoptosis (280) and that CCL22 expression is

correlated to TNFα activation (281). Therefore, it may be possible that CCL22 is a mediator in TNFα induced chondrocyte apoptosis, yet further experiments will be required to examine this directly. Although the observed differences in cytokine profiles between post-injury and uninjured young adults did not associate to significant differences in clinical parameters, this is expected since the patients are young and not yet showing symptomatic OA. This group will be ideal for further longitudinal follow-up.

Overall, the findings present in rat models and human OA strongly suggest that CCL22 could play a role in joint pain and initiation of cartilage degeneration.

5.6 Conclusion

In this study, the associations between cytokines in previously injured and uninjured participants were compared using network analysis. The overall cytokine association networks were significantly different, suggesting that injury had altered the inflammatory environment, which might contribute to joint degeneration. CCL2/MCP1, CCL22 and TNFα were the most different cytokines between two groups. Moreover, no association between cytokine expression knee injury history, self-reported pain symptoms and/or MRI-defined OA was found. In a rat model of OA CCL22 was correlated with pain and structural changes to the cartilage and CCL22 expression was found in damaged cartilage in addition to apoptotic chondrocytes. This result was validated in human cartilage and in isolated human chondrocytes, were CCL22

treatment induced apoptosis in a dose dependent manner. We propose that CCL22 is a novel potential biomarker in the earliest stages of cartilage damage and may also play a functional role in the degeneration of the articular cartilage through induction of chondrocyte apoptosis.

Chapter Six: CCL22 Induces Inflammatory Changes in Synovial Fibroblasts from Healthy Donors but Not Patients with Osteoarthritis

6.1 Abstract

Background: Synovium inflammation (synovitis) is commonly observed in the joints of patients within the earlier stages of OA. Synovitis is also found to be strongly associated with OA pain and progression of the disease. In previous studies, we have demonstrated that the chemokine C-C motif chemokine 22 (CCL22) induces chondrocyte apoptosis *in vitro* and we also observed that its expression level in serum was correlated with OA pain. However, the effects of CCL22 on other joint tissues such as synovium remain unknown. Therefore, in this study, our goal was to evaluate the effect of CCL22 on cells that make up the synovium, specifically, synovial fibroblasts; in terms of cell survival, inflammatory cytokine production and gene expression *in vitro.*

Methods: Synovial fibroblasts were harvested from synovial biopsies from healthy controls (n=10) and OA patients (n=9) and used in experiments before passage 5. Flow cytometry was employed to detect the presence of the receptor for CCL22, CC chemokine receptor 4 (CCR4). Cytokine protein expression and genome-wide gene expression were examined by Luminex Milliplex Assays and Affymetrix GeneChip Microarrays, respectively. qPCR was employed to validate the differentially expressed genes detected by microarray.

Results: No significant difference was observed in apoptosis rate and cell cycle between CCL22 treated synovial fibroblasts and untreated controls. CCL22 treatment significantly suppressed the anti-inflammatory cytokine IL10 at the protein level and promoted the pro-inflammatory protein S100A12 at the mRNA level in normal but not OA synovial fibroblasts.

Conclusions: CCL22 appears to be involved in the initiation of pro-inflammatory pathways in synovial fibroblasts. These results suggest that CCL22 may play a role in the initiation of synovitis through CCR4-independent pathways. How CCL22 regulates the expression of IL10 and/or S100A12 independently of CCR4 remains unclear and further studies in are needed to understand this novel CCL22 signaling pathway.

6.2 Introduction

Osteoarthritis is among the most common chronic diseases worldwide which can lead to disability (197). Although OA is characterized by progressive destruction to articular cartilage and subchondral bone, it is now considered as a whole joint disease involves the pathologic changes of many other joint tissues such as synovium inflammation (synovitis) (19, 282). Synovium is the inner surface of joint capsule which seals the joint cavity and holds synovial fluid. The cells within the synovium are responsible for producing synovial fluid lubricants (e.g. lubricin/PRG4 and hyaluronic acid) as well as filtering plasma as a source of nutrients for chondrocytes (283). Inflamed synovium in OA is often assoicated with histological changes (e.g. synovial lining hyperplasia) and leukocytes infiltration of the synovial lining (283). Furthermore, synovitis is also

strongly associated with the onset and progression of OA and it often observed in OA joints from the earliest to advanced stages of the disease (284). Synovitis is also strongly associated with OA pain, with the synovium being a highly innervated tissue compared to the non-innervated cartilage (285).

In previous studies, our lab and others have described an association between OA pain, cartilage degeneration and serum levels of C-C motif chemokine 22 (CCL22) (286). It is widely accepted that CCL22 is a chemokine that acts on $CCR4^{+}$ cells including T cells, and dendritic cells (among others) (287). We have previously demonstrated that exogenous application of CCL22 to human chondrocytes induces apoptosis in a dose-dependent manner *in vitro*. We further demonstrated that chondrocytes present within OA cartilage (human and rat models) co-expressed CCL22 and cleaved Caspase-3 (a marker of apoptosis) (chapter five). These results suggested that, besides chemotaxis, CCL22 may also be involved in additional pathways that regulate and/or lead to the degeneration of cartilage and potentially other joint tissues such as synovium. Interestingly, in another study, we found no evidence of CCL22 staining in synovium from OA patients (chapter three), with an independent study demonstrating minimal CCL22 expression in OA synovium and no expression in normal synovium (288). Therefore, to address these potentially conflicting results, and to determine if CCL22 acts upon synovial fibroblasts; in the current study we evaluated the effects of CCL22 on synovial fibroblast survival, inflammatory cytokine production and their gene expression *in vitro*. We also sought to determine if there was any difference in these outcome measures between synovial fibroblasts isolated from normal joints or from the

joint of patients with clinically diagnosed OA.

6.3 Materials and methods

6.3.1 Human participants

This study protocol was approved by the University of Calgary Human Research Ethics Board (REB15-0005 and REB15-0880). All patients involved provided signed consent/assent the study was carried out in accordance with the declaration of Helsinki. Normal Group (n=10): Criteria for control cadaveric donations were an age of 18 years or older, no history of arthritis, joint injury or surgery (including visual inspection of the cartilage surfaces during recovery), no prescription anti-inflammatory medications, no co-morbidities (such as diabetes/cancer), and availability within 4 hours of death. Knee Osteoarthritis (n=9): Inclusion criteria was based on a diagnosis of OA performed by an orthopedic surgeon at the University of Calgary based on clinical symptoms with radiographic evidence of changes associated with OA in accordance with American College of Rheumatology (ACR) criteria. Radiographic evidence of OA of any compartment of the knee with collapsed or near collapsed joint space of any compartment of the knee.

6.3.2 Synovial fibroblast derivation

To obtain synovial fibroblasts for analysis, two biopsies (approximately 5mm in diameter) were obtained from each donor and placed in 1.5mL tubes with 1xDPBS

(ThermoFisher) added to keep the tissues hydrated. Each synovial membrane biopsy was then roughly minced with a sterile razor blade and digested, at 37^0C, for an hour and a half with filtered type IV collagenase (Sigma) at a concentration of 1 mg/mL in heat-inactivated FBS (ThermoFisher).

The resultant cell suspension was filtered at 70µm (ThermoFisher) and centrifuged at 5000 rpm for 6 minutes. The resultant cell pellet was washed twice with 1 ml of 1xDPBS. Then cells were expanded in T25 culture flasks (Primaria, Corning/ThermoFisher) in media containing DMEM F12, 10% FBS, 1% Non-essential Amino Acids, and 1% Anti-anti (all ThermoFisher). Flasks were passaged when cells reached 80% confluence and all outcome measures were performed on cells before passage 5.

6.3.3 Cell cycle analysis by flow cytometry

Human synovial fibroblasts were plated at 50,000 cells per well in 6-well plates for each condition, allowed to adhere overnight, and treated with the respective condition. After treatment, the cells were fixed by suspending in ice cold PBS and vortexed gently while cold 70% ETOH was added dropwise to prevent clumping. The solution was stored overnight at 4ºC. Cells were then washed twice with PBS, pelleted and stained with 50 µl of 100 µg/ml ribonuclease (Sigma-Aldrich, St. Louis, Missouri) and 200 µl of 50 µg/ml propidium iodide (Sigma) and incubated for 30 minutes at room temperature. Samples were then run on a BD LSR II Flow Cytometer and analyzed

using ModFit software.

6.3.4 Cytokine expression analysis

Cells were plated in 12 well Primaria dishes 24 hours before cytokine treatment. CCL22 was added, so that the final concentrations 0.2ng/ml (N) and 3ng/ml (OA), which were the mean CCL22 concentrations in synovial fluid *in vivo* from normal control (N) and OA (OA) patients respectively based to our previous study (289). TNFα with a concentration of 5pg/ml were used as a positive control. Cells were then incubated for 24 hours after cytokine treatment and culture media were collected for cytokine profiling analysis. Cytokine profiling analysis was performed by Eve Technologies (Calgary, AB Canada) using the Milliplex MAP Human Cytokine/Chemokine Panel (Millipore), according to the manufacturer's instructions. All samples were assayed at least in duplicate and prepared standards were included in all runs. The following cytokines were examined by Luminex in this study: GM-CSF, IFNy, IL-1B, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p70), IL-13, MCP-1 and TNFα. The sensitivities of these makers range from 0.1 – 10.1 pg/mL (average 2.359 pg/ml) and the inter-array accuracies range from 3.5% – 18.9% coefficient of variation (average 10.7%).

6.3.5 Micro-Array analysis

RNA was extracted using Trizol Reagent (ThermoFisher) according to the manufacturer's protocol. Total RNA was purified with RNeasy Plus Micro Kit (Qiagen)

to remove genomic DNA. The RNA integrity number (RIN) was measured with Agilent RNA 6000 NanoChips on a 2100 Bioanalyzer (Agilent Technologies). The quantity was measured with a NanoDrop 1,000 (NanoDrop Technologies). A total of 300 ng of each RNA sample with RIN higher than nine was labeled with GeneChip Whole Transcript (WT) Sense Target Labeling Assay (Affymetrix) and hybridized to Affymetrix GeneChip Human Gene 1.0 ST Arrays at 45_C for 16 hours. Arrays were stained and washed on Affymetrix GeneChip Fluidics 450 following the manufacturer's protocol and scanned with an Affymetrix GeneChip Scanner 3000 7G System.

6.3.6 Micro-array data analysis

Statistical significant analysis was carried out on Transcriptome Analysis Console (TAC) Software (ThermoFisher). The fold change between normal and OA samples was based on the $p<0.05$ from a T-test (Asymptotic and Benjamini Hochberg FDR).

6.3.7 Relative quantification of gene expression

Total mRNA from each well was extracted and purified using Trizol in accordance with the manufactures instructions (ThermoFisher) and converted into cDNA according to the High Capacity cDNA Reverse Transcription Kits protocol (Applied Biosystems). Two microliters of cDNA was added to a 96 well qPCR plate along with S100A12 TaqMan® validated probes/primers and TaqMan® Universal PCR Master Mix. In addition, an 18S RNA probe/primer was used as an endogenous control. Two replicates

of each time point and control sample were performed, and all cDNA used was from the same corresponding replicate to reduce variability.

6.3.8 Data analysis

SPSS 25 (SPSS, Inc., Chicago IL) was used for the statistical analysis. Paired t-test (treatment vs. negative control) was used to compare individual cytokine expression. Two sample t-test was used for all the other univariate comparisons when paired t-test was not applicable. Multivariate analysis of variance (MANOVA) was used to compare cytokine profiling between treatment and control. Functional annotation was performed through DAVID (http://david.abcc.ncifcrf.gov/) for GO terms.

6.4 Results

6.4.1 Apoptosis and cell cycle analysis

Since we previously found that CCL22 induced apoptosis in chondrocytes in a dose dependent manner, we examined if the same range of CCL22 (0.2ng/ml (N) or 3ng/ml (OA)) induced apoptosis or alterations to the cell cycle in synovial fibroblasts derived from normal (n=3) or OA (n=3) patients. There was no significant change in the percentage of apoptotic cells after CCL22 treatment in normal or OA cell lines compared to the untreated control, while an increase in apoptosis was observed in OA cells after TNFα treatment (**Figure 6-1**). In normal synovial fibroblasts treated with 0.2ng/ml CCL22, there was a trend towards fewer cells observed in the G1 and G2 phase with a corresponding increase of cells in the S phase, compared to control.

However, none of the differences reached statistical significance (**Figure 6-1**). TNFα treatment did not have an impact on cell cycle.

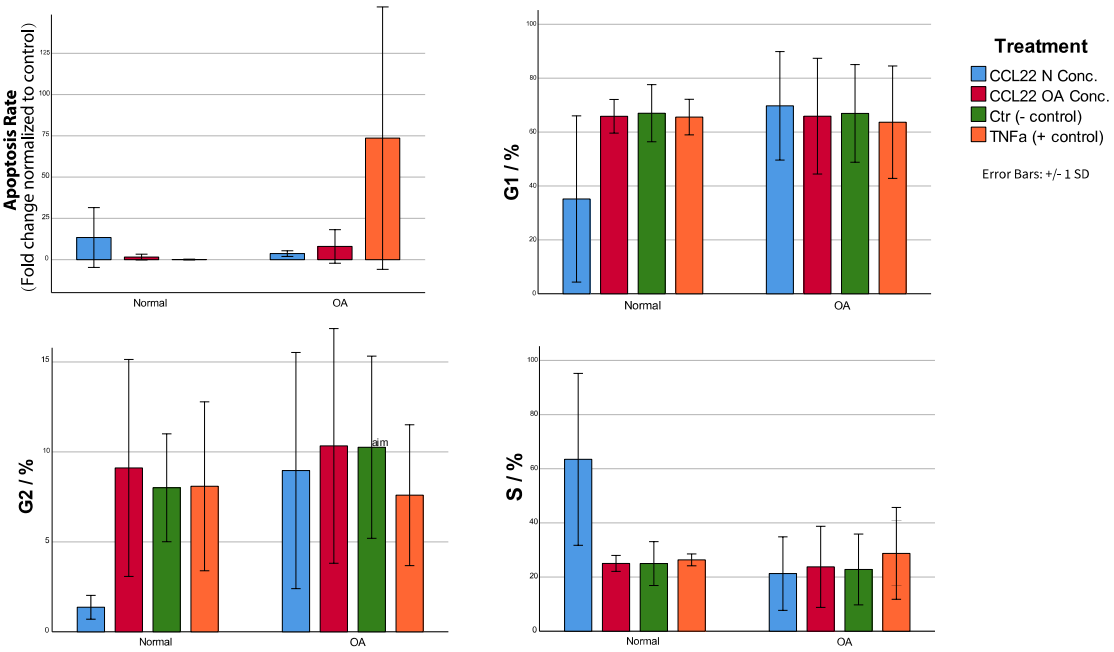


Figure 6-1. Apoptosis and cell cycle analysis after CCL22 treatment in normal (n=3) and OA (n=3) synovial fibroblasts. Without CCL22 (or TNFα) treatment, no differences were observed in the number of cells undergoing apoptosis or percentage of cells in each stage of the cell cycle between normal or OA synovial fibroblasts. While CCL22 (or TNFα) treatment did not significantly affect the cell cycle or induce apoptosis, a trend of increased apoptosis and decreased percentage of cells in G2 (with corresponding increase in S) was observed in the lower concentration CCL22 group (N). No effects were observed with the higher CCL22 concentration group (OA).

6.4.2 Cytokine expression analysis

Normal (n=7) and OA (n=6) synovial fibroblast cell lines were included for cytokine expression analysis. OA and normal synovial fibroblast demonstrated similar intense response to TNFα treatment as expected. With TNFα treatment, three cytokines were significantly overexpressed in normal cells: IL-8 (>2000-fold, p=.004), GM-CSF (>30-

fold, p=.007) and TNFα (>30-fold, p=.001). In OA cells, IL-8 (>4000-fold, p<.001), GM-CSF (>30-fold, p<.001) and TNFα (>60-fold, p=.001), MCP-1 (>400-fold, p=.001) were significantly increased (**Figure 6-2**). It should be noted that the increases in TNFα expression have been considered in relation to the amount of TNFα added (e.g. the values reported have had the 5pg/ml of TNFα subtracted before analysis). CCL22 treatment did not elicit a similar dramatic change in cytokine expression, however, a differential response was observed between normal and OA fibroblasts. In normal cells treated with the higher concentration of CCL22 (OA), the cytokine expression as a whole, was significantly altered (p<.001), however, this was not observed in OA fibroblasts (p=.842). In regard to specific cytokines, a trend was observed in the expression of IL8, IL4 and IL10, as the levels were increased in OA cells but suppressed in normal cells (relative to their respective negative controls), however, only IL10 levels reached significance (p=.047). Neither OA nor normal synovial fibroblasts responded to the lower concentration of CCL22 (N).

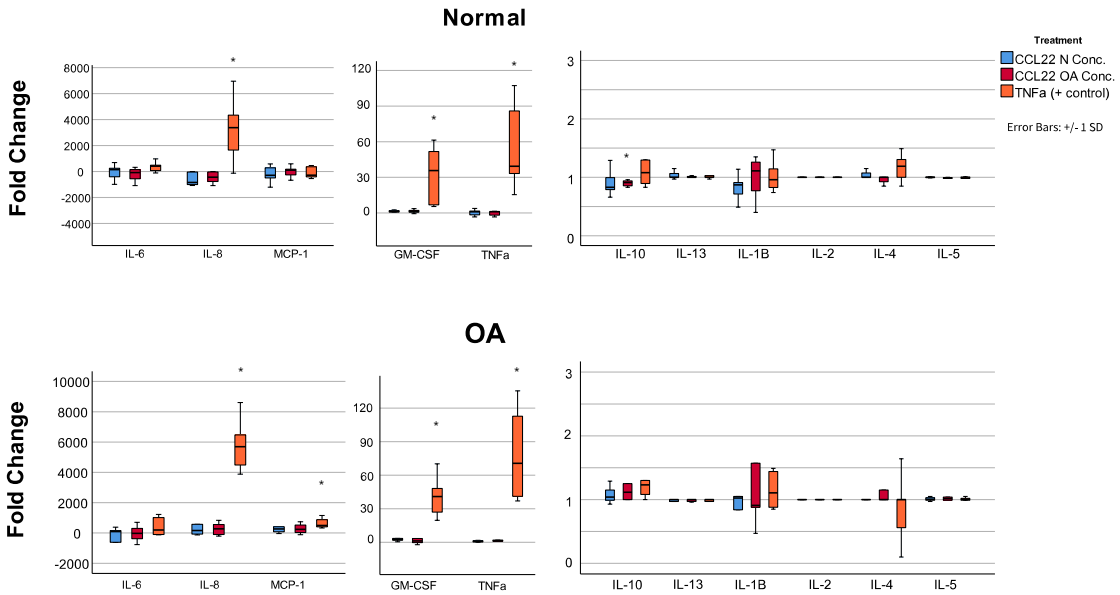


Figure 6-2. Cytokine expression response to CCL22 treatment. While TNFα treatment increased the expression of a number of cytokines examined, CCL22 treatment did not increase the expression of any cytokine examined, and both concentrations tested had no effect on the cytokine expression in OA synovial fibroblasts. In normal synovial fibroblasts, the higher concentration treatment group (OA), significantly decreased the expression of IL10. *p<0.05.

6.4.3 Transcriptomic analysis

Since it was observed the CCL22 treatment could down regulate the expression of IL10 in normal synovial fibroblasts. Microarray analysis was used to explore the cell responses to CCL22 treatment in terms of genome-wide gene expression. Twelve genes were found significantly differentially expressed after normal synovial fibroblasts (n=3) were treated with the high concentration of CCL22 (OA) as shown in **Table 6-1**. No differentially regulated genes were detected in OA synovial fibroblasts (n=3) treated with high concentration of CCL22. These genes observed in normal fibroblasts treated with CCL22 were not significantly enriched in any GO term or signaling pathway (**Table 6-2**). While no changes in expression of IL10 was

observed with CCL22 treatment, S100A12, a protein associated with the pathogenesis of OA was significantly increased after CCL22 treatment. RT-qPCR was used to validate the effect of CCL22 on S100A12 expression. In accordance with the microarray results, S100A12 was significantly elevated ($p<.001$) in normal cells after CCL22 treatment (**Figure 6-3**). Meanwhile, S100A12 did not respond to CCL22 treatment in OA synovial fibroblasts.

Table 6-1. Differentially expressed gene in normal synovial cell 24h after CCL22 treatment.

Fold Change	Gene Symbol	Description
-12.85	LINC00652	long intergenic non-protein coding RNA 652
-6.47	VTRNA1-1	vault RNA 1-1
-5.39	SNORD58A; RPL17	small nucleolar RNA, C/D box 58A; ribosomal protein L17
-5.25	LOC102724208	LOC102724208
-2.14	ZNF443	zinc finger protein 443
-2.14	RBMY1B/D/E	RNA binding motif protein, Y-linked, family 1, member B/D/E
-2.09	IGKV2D-29	immunoglobulin kappa variable 2D-29
2.03	MGC39584	LOC441058
2.05	LOC646029	LOC646029
8.07	S100A12	S100 calcium binding protein A12
12.38	GRAP	GRB2-related adaptor protein
22.46	EYS	eyes shut homolog (Drosophila)

Table 6-2. DAVID functional annotation chart

Category	Term	Genes	P-Value	Benjamini
GOTERM_CC_DIRECT	cytosol	GRAP, S100A12, RPL17	9.50E-02	5.90E-01
GOTERM_CC_DIRECT	nucleus	S100A12, RPL17, ZNF443	2.20E-01	6.70E-01

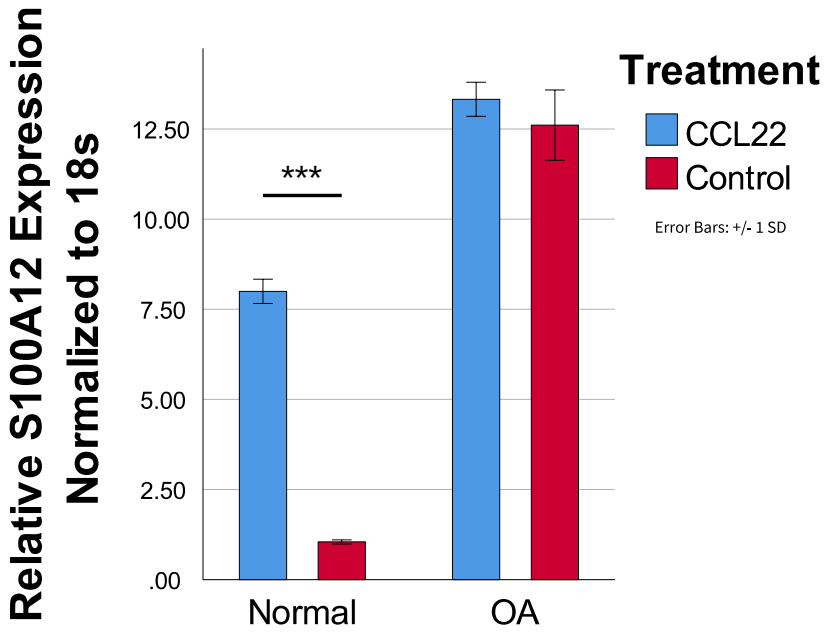


Figure 6-3. S100A12 expression in CCL22 treated synovial fibroblasts. Normal (n=3) and OA (n=3) synovial fibroblasts were treated with the high concentration of CCL22 and the expression of S100A12 was quantified using RT-qPCR. While CCL22 treatment significantly increased the expression of S100A12 in normal fibroblasts, no effect was observed in OA fibroblasts. *p<0.001.

6.4.4 Absence of CCR4 on synovial fibroblasts

Since CCR4 is the only known receptor for CCL22 and previous studies have demonstrated the absence of CCR4 in normal human synovium (288), flow cytometry analysis was used to determine if CCR4 was expressed on the cell surface of normal (n=2) or OA (n=2) synovial fibroblasts. Interestingly, none of synovial fibroblast cell

lines examined (normal or OA) demonstrated surface expression of CCR4 (**Figure 6-4**).

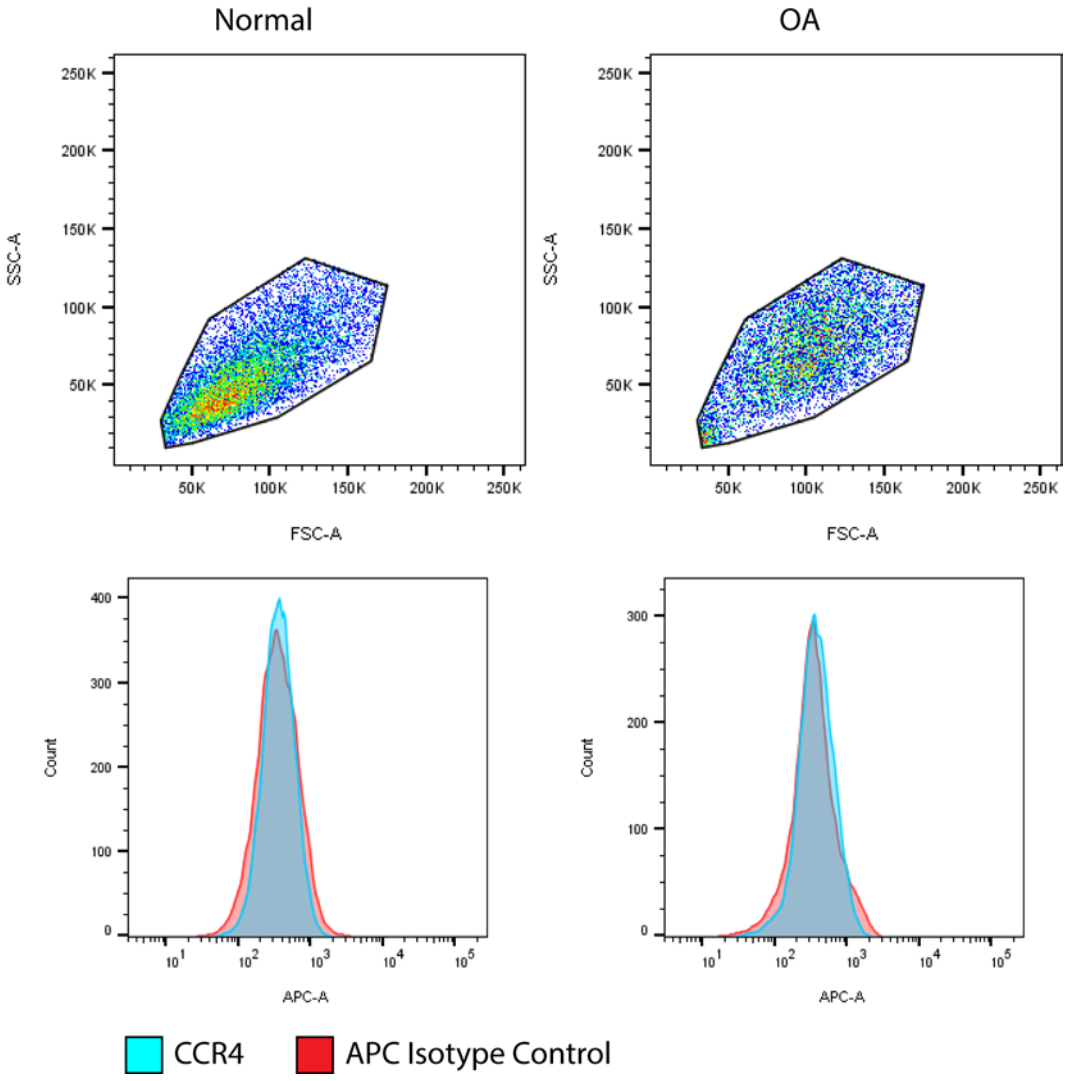


Figure 6-4. Flow cytometric analysis of CCR4 on synovial cells. None of the cell lines examined demonstrated CCR4 an increase in positive staining compared to the negative control.

6.5 Discussion

The role of cytokines in the pathogenesis of OA has become increasingly recognized (290). Major pro-inflammatory cytokines such as TNFα and IL1β have a direct and destructive impact on articular cartilage not only by promoting the expression of

cartilage extracellular matrix proteinases (e.g. matrix metalloproteinases), but also by through induction of chondrocyte apoptosis (291-293). In the last decade, a variety of new cytokines had been found associated with tissue degeneration and pain in OA (294). In previous studies from our group and other, the chemokine CCL22 is known to be elevated in synovial fluid and serum from OA patients and is correlated with OA pain (286, 288, 289). CCL22 (Chemokine ligand 22), also named macrophage derived chemokine (MDC), is commonly produced by macrophages, activated B lymphocytes and dendritic cells. CCL22 has a complex role in inflammation and is recognized as a potent chemotactic molecule, recruiting both anti- and pro-inflammatory immune cells (275, 276). It has been recently reported by our lab that CCL22 can induce apoptosis in human chondrocytes *in vitro* and it is expressed in chondrocytes throughout the progression of OA in a rat model. These results suggest that it might play a role in the initiation of cartilage degeneration in OA. Therefore, the aim of the present study was to investigate if CCL22 could also trigger the inflammation or apoptosis in synovial fibroblasts from healthy donors and OA patients. While, we did not observe an increase in apoptosis in synovial fibroblasts with CCL22 treatment, we did find that CCL22 regulated the expression of inflammatory cytokines in normal (but not OA) synovial fibroblasts. We also observed that CCL22 is an inducer of S100A12 expression, but this effect was only observed in normal fibroblasts as OA fibroblasts already demonstrate increased expression of S100A12.

Synovitis is often observed in the earliest stages of OA (295), however, it is not clear whether synovitis is primarily caused by systemic immune responses or occur

secondarily to joint tissue damage. Prior research suggests that once a given threshold of inflammation is reached within the joint, a variety of cytokines including IL6 and TNFα in the inflamed synovial fluid could stimulate synovial cells to also produce inflammatory cytokines, magnifying the level of inflammation in the joint (296). In the current study, we found that CCL22 treatment significantly downregulated IL10 expression and upregulated S100A12 expression in normal but not in OA synovial fibroblasts. IL10 is an anti-inflammatory cytokine, capable of suppressing the immune response through a variety of mechanisms, including inhibiting the synthesis of pro-inflammatory cytokines such as IFNγ, and TNFα and GM-CSF (297). S100A12 is a pro-inflammatory cytokine-like protein. It is involved in OA development by up-regulating MMPs and activating the NF-κB pathway (56). The change of IL10 (down-regulation) and S100A12 (up-regulation) expression in normal synovial fibroblasts indicates that CCL22 might play in important role in initiating/promoting a pro-inflammatory response in synovium.

It is not uncommon for cytokines/chemokines to induce apoptosis (298), however, we found no significant change in the level of apoptosis, or any differential cell cycle regulation with CCL22 treatment. This might due to the fact that the incubation time was too short (24 hours) after treatment, yet we previously observed chondrocyte apoptosis in the same timeframe with using the same concentration of CCL22. It is also important to note that TNFα treatment significantly increased apoptosis only in OA cells. Therefore, there may be differences in the sensitivity of synovial fibroblasts to cytokine/chemokine induced apoptosis, and this may be related to the inflammatory

environment they have been previously exposed to *in vivo*. Furthermore, it may be possible that CCL22 induced apoptosis is indirect in synovial fibroblasts since it has been previously demonstrated that S100A12 can induce apoptosis (299). Therefore, longer time-points may have resulted in an increase in apoptosis if this effect is working through the S100A12 pathway.

Since CCR4 is the only known receptor for CCL22 (287), and we observed changes to both IL10 and S100A12 expression after CCL22 treatment, we were surprised to find that neither normal nor OA synovial fibroblasts expressed CCR4 on the cell surface. These results are therefore truly conflicting and difficult to reconcile. While we most likely had a mixed population of cells in our fibroblast cultures, our flow cytometry data demonstrated no CCR4 positive cells (regardless if they were synovial fibroblasts or not), suggesting that there was not a contaminating population of CCR4 positive cells reacting to the CCL22. Therefore, we suggest that there may be additional receptors or co-receptors for CCL22. While we didn't find any evidence for this in the literature, it is possible that CCL22 has additional binding partners other than CCR4 and this should be researched in the future.

Unlike rheumatoid arthritis (RA), the inflammation in OA is often low-grade and confined within affect joints. Therefore, inflammation inhibitors targeting at the major pro-inflammatory cytokines (e.g. TNFα, IL6 and IL1B) that demonstrate benefit for RA patients, have not demonstrated significant efficacy in OA, at least at the early stage of the disease. Indeed, studies showed that many anti-inflammatory drugs directed at these cytokines had a less dramatic impact in OA patients than RA patients, and could lead to

many side effects such as increased risk of infection (300, 301). These results may suggest that these cytokines that drive the inflammatory process in RA, could be merely a secondary phenomenon followed by tissue damage or other inflammation pathways in OA. In this study, we found that CCL22 could induce inflammation by inhibiting IL10 and promoting S100A12 expression in normal synovial fibroblasts and on the surface, this result could be interpreted as driving a pro-inflammatory response. However, we concede it is unlikely that CCL22 is driving/perpetuating a chronic synovial inflammatory response as CCL22 failed to stimulate an increase in inflammation in OA synovial fibroblasts similar to TNFα. Therefore, additional studies in pre-clinical model including transgenic mice with altered CCL22 expression are needed to validate this hypothesis.

6.6 Conclusions

In this study, we investigated the effect of CCL22 on synovial fibroblasts from OA patients and healthy controls. We found CCL22 significantly suppressed the anti-inflammatory cytokine IL10 and promoted the pro-inflammatory protein S100A12 in normal but not OA synovial fibroblasts. We therefore propose that CCL22 could be playing an important role in the initiation of synovitis and therefore its potential as a biomarker as well as a therapeutic target in the early stage of OA should be further researched.

Chapter Seven: Discussion and conclusions

7.1 Discussion

In this chapter, the key findings of the thesis are presented and discussed. Some of the challenges of performing prospective OA studies in humans and therefore the rationale for high-throughput data exploratory followed by *in vivo* and *in vitro* experimental validation are then discussed. The advantages and limitations of the study, including its potential clinical application are discussed, and finally, potential future directions are presented and discussed.

7.2 Summary of Key Findings

The overall goal of this thesis was to characterize serum inflammatory/cytokine profiling in participants who are at a high risk of developing OA, for the purpose of developing a biomarker indicative and possibly predictive of early stage (pre-radiographic) OA in a youth knee injury cohorts. To achieve this goal, serum cytokine profiles in a number of distinct cohorts were analyzed and a candidate biomarker was selected and validated using *in vivo* and *in vitro* methodologies. Specifically, in this thesis, firstly, exploratory data analysis was applied on cytokine profiling data including comparing serum cytokine profiles between patients with hip OA, knee OA, participants with high risk of developing OA (knee injury) and healthy controls, and examining the association between clinical measurements and OA risk factors and serum cytokines. The analysis confirmed the value of serum cytokines profile in

differentiating these groups. Then focus was narrowed down to one specific candidate cytokine-CCL22 through the use of a novel computational method. Lastly, the biological role of CCL22 in the onset of OA was validated both *in vivo* (rat model of OA) and *in vitro* (human cartilage tissue and synovial fibroblast). The major findings are:

(1) Serum cytokine profiles were significantly different between the cohorts examined in this thesis: In middle-aged and older adults, cytokine profiles were found to be significantly different between OA patients vs. healthy controls. Within OA patients, EGF, FGF2, MCP3, MIP1α, and IL8 were expressed differently according to affected joints (knee vs. hip); in young adults, serum cytokine profiles were significantly different in terms of cytokine associations (but not expression) in individuals with vs. without knee injury 3-10 years previously in their youth. MCP1, CCL22 and TNFα were the most altered cytokines in terms of their associations with other cytokines.

(2) A potential early OA biomarker and therapeutic target - CCL22 was identified. In hip OA patients, CCL22 was associated with pain and found to be present in synovial fluid and synovial membrane. In knee OA patients, CCL22 was found present in the cartilage and able to induce apoptosis in articular chondrocytes *in vitro*. Moreover, CCL22 significantly suppressed the anti-inflammatory cytokine IL10 and promoted the pro-inflammatory protein S100A12 in synovial fibroblasts *in vitro* from healthy knee joints. In a rat model of early OA, CCL22 was present chondrocytes in areas of cartilage lesions and co-localized with

markers of apoptosis. A summary of the potential roles that CCL22 played in the pathogenesis of OA is shown in **Figure 7-1**.

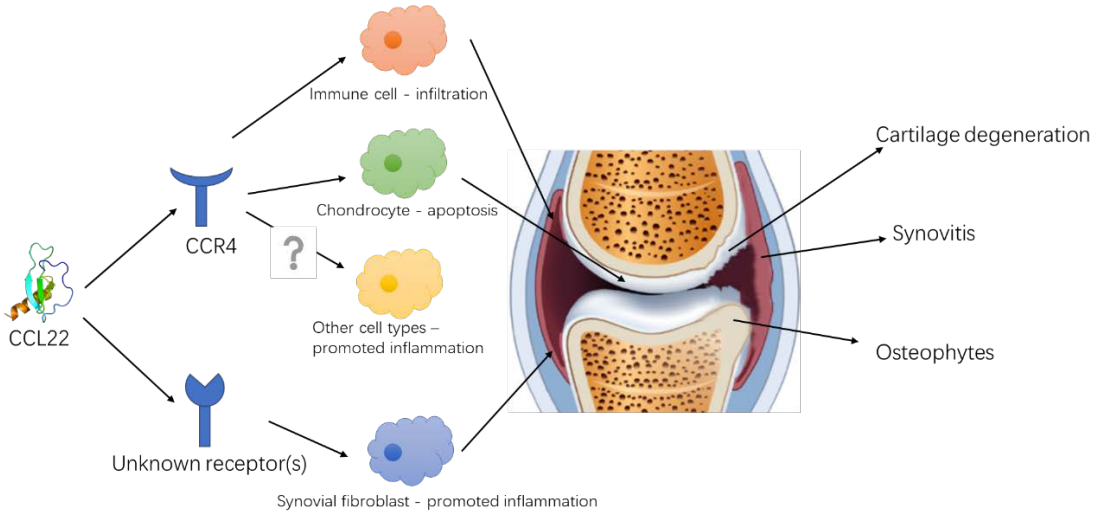


Figure 7-1. CCL22 may act on multiple tissue in the onset and development of OA. While some of these effects are likely driven through a CCR4 mediated pathway; the actions of CCL22 in the synovium appear to be CCR4 independent.

7.3 Strengths and Limitations

In humans, the development of clinically diagnosable OA usually takes years or even decades. While ideal, it is impractical for a research group to conduct longitudinal studies following the same cohort for such a long period of time until a sufficient percentage of the cohort develops OA to ensure the study is adequately powered. On the other hand, many animal models of OA demonstrate a rapid progression of the disease, typically with significant cartilage damage within weeks (302). However, evidence has showed that animal models of OA have different responses vs. humans and hence might not be truly representative of human OA and/or inflammatory diseases

that affect the joint (93). Therefore, in this thesis a strategy combining cross-sectional and longitudinal human cohorts was adopted with an attempt to validate the human findings *in vivo* in a pre-clincal rat model of OA. Based on the findings of the rat study, it was then attempted to validate these findings *in vitro* using cultured human chondrocytes and synovial fibroblasts. Notwithstanding that a strategy designed to cross validate our human and pre-clinical animal model data was employed, the current thesis is not without limitations. The perceived strengths and limitations of the thesis will be discussed in the following sections.

7.3.1 Study Strengths

One of the main study strengths was that this thesis work included in a number of distinct cohorts including clinically diagnosed OA, high risk of OA and healthy controls. The pathogenesis of OA is known to be multifactorial; aging, injury, and genetic predisposition may all act as contributing factors lead to the joint degeneration. Many studies have suggested that OA is a collection of different subtypes/phenotypes (303). Therefore, one of the strengths that comes from including multiple cohorts with different backgrounds is that the result of the study may be more generalizable to the larger population and/or patient population. While previous studies have also demonstrated differentially expressed biomarkers in hip compared with knee OA (304). The current thesis is novel since OA patients with different affected joints (hip and knee) were included and discriminated as hip or knee when comparing OA with control (non-OA) groups. Finally, in this thesis, individuals at different stages of OA were induced,

from those who are at high risk but have not yet developed the disease to the end-stage OA patients who underwent total knee replacement. The high risk cohorts are crucial in finding these candidate biomarkers in the earliest stages of the disease and thereby facilitating the developing of interventions for early OA. Without reliable biomarkers that can accurately diagnose pre-clinical OA, it is difficult to develop cohorts to test novel interventions in patients in the early stages of the disease. Therefore, stringently developed cohorts of individuals at high risk of developing OA may hold vital clues for understanding the early/pre-structural changes in the disease; which may help in the detection and development of interventions for early OA.

Another strength of this thesis, was that this study utilized a Luminex multiplex array platform which allowed us to quantify 41 cytokines per sample per 96 well plate. The Luminex platform has the benefit of being more efficient than traditional ELISA approach which is commonly applied in similar studies. The Luminex approach significantly expanded the scope of the study by increasing the number of cytokines that could be examined, this was essential to since this study required a screening approach to identify candidates for further examination. Compared to other high-throughput techniques such as microarray and MS, Luminex is more sensitive, with a lower detection limit of 0.1-10.1 pg/mL (with an average of 2.359 pg/ml) and therefore was superior in detecting (and fully quantifying) low-abundant biomarkers such as cytokines (cytokine concentration in serum can be as low as 1pg/ml while the sensitivity of MS is usually >10 pg/mL).

Another strength of the current thesis, is that our studies employed exploratory data analysis (EDA) with experimental validation. The purpose of EDA is to develop a detailed understanding of complex data sets such as non-uniformed clinical measurements and cytokine profiles from heterogeneous cohorts included in this study. Different from the hypothesis-driven analysis, which requires the researcher to be very specific about the analyses they wish to perform, EDA is open and flexible, similar to the detective work which involves repetitively examining the data, generating of hypotheses and assessing how well the tentative theories fit the data (305). For complex data sets such as biomedical data, without EDA, one may only find the information they look for, but will never discover insights that lie beyond prior knowledge. However, many statisticians argue that EDA can become "data dredging/fishing" which may damage scientific research if used incautiously (306). Therefore, EDA was applied in the first step of data analysis to maximize our potential/creativity in discovering patterns in data, followed by experimental validation to support the novel findings generated through EDA.

7.3.2 Study Limitations

A limitation of this thesis is that completely uniform clinical information was not available for each cohort. For example, the clinical measurements (e.g. pain and functionality) were only available for hip OA cohorts and PrE-OA cohorts; the age information for each patient sample in the mid-aged control cohort was unavailable due

to ethics constraints. Missing data points were also a limitation. Some participants in PrE-OA cohort were missing MRI information or dropped out during the study period. Therefore, it was not possible to run all analysis in all cohorts, making it impossible to conduct parallel comparison across cohorts. Additionally, many factors which could confound cytokine concentrations, such as individual characteristics (e.g. race, disease history, diet among other factors) were not collected.

During the study period, none of the high-risk participants in PrE-OA developed clinically defined OA. Consequently, the inflammatory profile change in the whole transition process from beginning (normal) to end (disease) was not able to be monitored/quantified. However, it was still possbile to identify significant different cytokines between with vs. without a history of injury and conducted downstream experiments based on the finding.

Consistently, all these findings supported that CCL22 plays an important role in the onset of cartilage degeneration. In human cohorts, CCL22 was found significantly different between individuals with and without a history of injury in terms of connectedness with other cytokines. It also significantly correlated with pain in hip OA patients. In the rat model of early OA, CCL22 was also found correlated with pain. Moreover, CCL22 was present not only in severely damaged joints in rats, but also in the cartilage before the visible damage occurred. Furthermore, CCL22 expression was observed in chondrocytes undergoing apoptosis (cleaved caspase 3 positive cells), with almost all apoptotic cells being positive for CCL22. However, with many of these results being observational/descriptional in nature, the cause and effect relationship

between CCL22 and cartilage degeneration/OA still remains elusive. Even though a functional role of CCL22 in the induction of apoptosis of human chondrocytes and the induction of a pro-inflammatory response in human synovial fibroblasts was demonstrated, these results do not necessarily imply that CCL22 will lead to cartilage degeneration *in vivo*.

Another limitation is that this thesis did not clarify the source of CCL22 in the initial stage of OA. Although there was intensive positive CCL22 staining observed in rat cartilage (**Figure 5-4**), it does not necessarily mean that chondrocytes produce CCL22. In fact, in OA or healthy patients, the concentration of CCL22 was not significantly higher in synovial fluid than that in serum (**Figure 3-4**), indicating that the majority of CCL22 in serum was unlikely coming from the joints. Previous studies have demonstrated that in addition to macrophages and dendritic cells, epidermal cells such as keratinocytes are also a main source of CCL22 (307). Therefore, it is possible that the majority of CCL22 in circulation and synovial fluid is derived from these cells outside the joint environment. Future studies are needed to test this hypothesis.

7.3.3 Significance

Currently the clinical diagnosis of osteoarthritis (OA) is typically only made only if the patient is symptomatic (e.g. joint pain, loss of mobility). However, the difficulty of using symptoms to define the presence of early-stage OA is that these symptoms develop once the disease is advanced and possibly irreversible. In this thesis, a novel candidate early OA biomarker (CCL22) has been identified that may have promise to

identify patients in the early-stages of OA, before irreversible joint damage has occurred. If this biomarker can be validated in an independent cohort, it may have significant clinical potential as it could be easily added to a routine blood test.

This thesis has also added to our understanding of the effect of an intra-articular knee injury on systemic inflammatory cytokine expression that may influence the future onset and/or progression of OA in this high risk cohort. By improving our understanding inflammatory factors in disease pathogenesis, this study have the potential to aid in increasing our fields ability to identify patients at greatest risk of OA; increase the accuracy of diagnosing early OA; and measuring treatment efficacy within pre-radiographic OA cohorts.

Further, since it was demonstrated that CCL22 may also play a functional role in the onset of OA, this knowledge may be useful as guidance to develop new effective management/treatment strategies for joint injuries with the purpose to prevent or postpone OA development. For example, if a drug targeting CCL22 could be developed and demonstrated efficacy for slowing the progression of OA; this could impact on how health care is delivered to early OA patients. Furthermore, it could also be possible that the inflammatory profiles themselves could be used to guide health care decisions. The need of arthroplasty surgery might be prioritized within individuals that have a biomarker profile similar to that of patients that were at the point of requiring joint replacement vs. those with clinically diagnosed but less severe OA.

7.1 Future Directions

7.1.1 Follow up of PrE-OA cohort

In the PrE-OA cohort, the association between cytokine profiles and pain was not significant. This is expected since the patients are still relatively young (e.g. mid 20's) and not yet showing symptomatic OA. However, a small portion of participants were diagnosed with MRI-defined OA. We have started to focus on these individuals and in the **Appendix**, we demonstrate that participants with vs. without MRI-defined OA present with different cytokine profiles (when comparing the cytokine association networks). One question that remains unsolved due to the small number of participants with MRI-defined OA in the current thesis, is what is the value of cytokine in predicting MRI-defined OA (or, whether cytokines and MRI-defined structural changes are correlated) for each individual? Further, many studies have reported that MRI can detect minor cartilage lesions which could be representative of early OA, while others argue that the minor tissue damage was also common in the general population of a similar age (241-243). The other interesting question is what is the value of combining cytokines profiles and MRI results in detecting early OA? Although we were able to detect changes over time in cytokine profile in the PrE-OA cohort, the changes were not in the direction of either the control or clinically diagnosed OA groups (**Appendix**). Are there any another covariate (i.e., age, sex) influencing serum cytokine profile? As future work, the PrE-OA cohort will be ideal for the longitudinal follow-up to address these open questions.

7.1.2 The mechanism of CCL22 promoting joint tissue degeneration

Base on the results of this thesis and previously published studies, it could be hypothesized that CCL22 might play multiple roles mediated through different pathways in different cell types and these functions may contribute to the degeneration of cartilage, and OA pain.

In chondrocytes

Although the *in vitro* experiments showed that CCL22 did not promote the expression of TNFα in synovial fibroblasts, it has been previously demonstrated that in chondrocytes, that CCL22 expression is correlated with the activation of TNFα which plays a role in chondrocyte apoptosis (280, 281). Giving the fact that in this study, CCR4 expressed in chondrocytes but not in synovial fibroblasts, it may be possible that CCL22 is a mediator in TNFα induced chondrocyte apoptosis through the CCR4 pathway. Further experiments will be required to examine this hypothesis directly.

In synovial fibroblasts

In normal synovial fibroblasts, it was observed that CCL22 significantly suppressed the anti-inflammatory cytokine IL10 and promoted the expression of pro-inflammatory factor S100A12. Since CCR4 was not found expressed on the cell surface, there may be additional receptors or co-receptors for CCL22. While no evidence for this is found in the literature, it is possible that CCL22 has additional binding partners other than CCR4 and this should be studied in the future. It should also be noted that CCL22 had

limited effect on OA synovial fibroblasts. This might indicate that this "potential binding partner" may no longer be expressed in synovial fibroblasts after the onset of OA; or potentially that these pathways are already activated in OA and therefore are insensitive to further activation. However, these speculations require further investigation.

In other cell types

Although it is widely accepted that the chondrocyte is the main resident cell type in articular cartilage (36), recent evidence has demonstrated that cartilage may possibly contain other cell types such as mesenchymal stem cells (MSCs) (308). MSCs was believed to play an important role in tissue regeneration as well as regulate the inflammatory response (309). Previous studies have shown that a small percentage of MSCs expressed CCR4, indicating that CCL22 might also be able to regulate this cell type. If that is the case, and tissue resident MSCs (either in cartilage or synovium) contribute to cartilage repair in some form, then it is possible that CCL22 also acts on MSCs to disrupt this function, thereby promoting degeneration by impeding the regenerative/reparative role of MSCs (310).

One of the best ways to verify the role of CCL22 in cartilage function and/or OA is using a transgenic knockout animal model, and in this case a conditional knockout mouse in where CCL22 could be deleted with chondrocytes alone would be preferable, since the function of CCL22 throughout the body is not well understood. Unfortunately, to our best knowledge, there is no conditional CCL22 knockout mouse currently available. Interestingly, a recent study using $CCR4^{-/-}$ mice and CCL17 gene-deficient

(CCL17$^{E/E}$) mice demonstrated the pivot role of the CCR4 pathway in cartilage destruction, development of osteophytes and pain in a collagenase-induced OA model (311). This study is directly related to this thesis since the gene for CCL22 is mapped to chromosome 16q13 and is near to another chemokine, TARC/CCL17 (312), which also signals through CCR4 (313). It is likely that these two chemokines evolved through gene duplication, but in humans they share only 37% amino acid sequence identity and are not equivalent in their expression or function, with CCL22 the stronger driver of CCR4 (314). This study on CCL17 and OA was a solid confirmation of our finding in terms of the role of CCL22 in the joint tissue degeneration and the development of OA pain. However, the authors failed to explain why knocking out CCL17 alone but not CCL22 had the same protective effect as knocking out CCR4, given that CCL22 is also a robust (and dominant) ligand of CCR4. Future studies using CCL17 and CCL22 double knockout mice would be an important step towards clarifying the relationship between CCR4 and CCL17/22 in the development of collagenase-induced OA. Moreover, it is also important to test if the CCR4-dependent pathway is also required in other OA models such as injury-induce OA.

As previously discussed, one of the downsides of using a mice model is that the inflammatory response in mouse might not be representative for human. In this thesis, the microarray analysis identified that S100A12 was elevated in CCL22 treated synovial fibroblasts. However, rodents do not have functional S100A12 gene (315). Therefore, CCL4 induced inflammatory response in synovial fibroblasts might be a human specific phenomenon and therefore a similar effect may not be observed in a

mouse model.

7.2 Summary

This thesis has generated knowledge regarding cytokine expression in a variety of patient cohorts, including clinically diagnosed hip and knee OA, high risk of OA and healthy controls. Further I have identified a potential biomarker, CCL22 which could be indicative of early stage (pre-radiographic) OA in knee injury cohorts. The latter part of this thesis attempted to validate the serum based findings and understand the biological role of CCL22 in the initiation and development of OA. CCL22 knock-out mice and/or alternative approaches are required in future studies to further validate the role of CCL22 as a diagnostic and therapeutic target in the early stages of OA.

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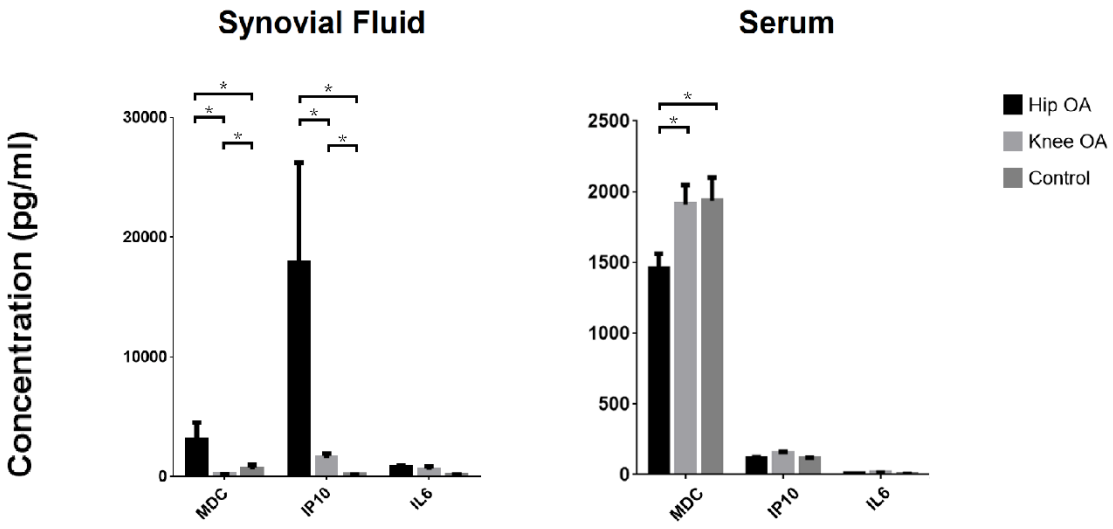
Chapter Nine: Supplementary Data



Supplementary Figure 9-1 Presence of MDC, IL6 and IP10 in Hip OA, Knee OA and control synovium, synovial fluid and serum not corrected for multiple comparisons. Serum and synovial fluid levels of MDC, IL6 and IP10 were examined in the three cohorts. * $p<0.05$. Abbreviations used in figure legend: MDC (macrophage derived chemokine), IL6 (interleukin 6), IP10 (interferon gamma-induced protein 10), OA (osteoarthritis).

Supplementary Table 9-1. Biochemical markers that have been reported to be correlated with OA pain

Marker	Pain Criterion	Population	OA Joint	Bodily Fluid	Sample Size	Citation #
CD163	NHANES-I		Knee	Synovial fluid, blood	184	(316)
CD14	NHANES-I		Knee	Synovial fluid, blood	184	(316)
NPY	Hideo Watanabe's knee scoring system-related pain score		Knee	Synovial fluid	100	(317)
COMP	AUSCAN pain		Hand	Serum	663	(30, 43)
IL15	WOMAC pain score	Chinese	Knee	Serum	226	(318)
IL6	VAS, JKOM-pain	Japanese	Knee	Serum	160	(319)
leptin	WOMAC and VAS		Knee, hip	Synovial fluid	219	(320)
hs-CRP	WOMAC		Knee	Serum	149	(321)
TNFα	WOMAC		Knee	Serum	149	(321)
ARGS	KOOS pain		Knee	Synovial fluid	141	(322)
CTXII	VAS	Japanese	Knee	Urine	47	(323)
NTX	VAS	Japanese	Knee	Urine	47	(323)
HA	VAS	Japanese	Knee	Serum	47	(323)
CPII	VAS	Japanese	Knee	Serum	47	(323)

Abbreviations used in table: CD (cluster of differentiation), NPY (neuropeptide Y), COMP (cartilage oligomeric matrix protein), IL (interleukin), hs-CRP (high-sensitivity C-reactive protein), TNFα (tumor necrosis factor alpha), ARGS (aggrecan neo-epitope fragment), CTXII (carboxy-terminal telepeptides of type II collagen), NTX (n-telopeptides of type 1 collagen), HA (hyaluronan), CPII (proCollagen II c-propeptide), NHANES (National Health and Nutrition Examination Survey), AUSCAN (AUStralian CANadian Osteoarthritis Hand Index), WOMAC (Western Ontario and McMaster Universities Osteoarthritis Index), VAS (Visual Analogue Scale), JKOM (Japanese Knee Osteoarthritis Measure), KOOS (Knee injury and Osteoarthritis Outcome Score).

Supplementary Table 9-2. Questionnaire scores of hip OA cohort

Questionnaire scores	K/L grade 3 (mean ± SD)	K/L grade 4 (mean ± SD)	P-value	Benjamini-Hochberg critical value
MHSF36	44.6 ± 8.1	52.3 ± 9.7	0.007**	0.007407
GHSF36	48.8 ± 6.8	54.2 ± 7.8	0.013*	0.014815
PCSSF36	32.8 ± 8.2	27.0 ± 5.7	0.018*	0.022222
MCSSF36	45.9 ± 10.8	54.9 ± 11.8	0.024*	0.02963
HOOSp6	2.5 ± 0.9	3.1 ± 0.9	0.034*	0.037037
HOOSp5	3.1 ± 0.7	3.7 ± 0.9	0.037*	0.044444
PFSF36	31.4 ± 9.4	25.5 ± 5.6	0.052	0.051852
HOOS	48.4 ± 12.9	41.1 ± 11.0	0.082	0.059259
HOOS Pain	32.1 ± 6.25	35.4 ± 5.0	0.083	0.066667
HOOS Pain factor 1	0.41 ± 1.14	0.16 ± 0.91	0.084	0.074074
HOOSp1	4.1 ± 0.7	4.5 ± 0.5	0.104	0.081481
HOOSp10	3.5 ± 0.6	3.8 ± 0.8	0.176	0.088889
HOOSp9	3.2 ± 0.8	3.5 ± 0.7	0.178	0.096296
BPSF36	34.3 ± 4.9	32.5 ± 6.2	0.184	0.103704
RPSF36	36.0 ± 10.7	32.1 ± 7.6	0.195	0.111111
RESF36	38.8 ± 14.7	43.6 ± 14.4	0.199	0.118519
HOOSp2	3.1 ± 0.9	3.4 ± 0.7	0.21	0.125926
HOOSp4	3.0 ± 0.8	3.3 ± 0.8	0.224	0.133333
SFSF36	37.1 ± 8.6	39.8 ± 11.8	0.33	0.140741
HOOSp8	3.0 ± 0.8	3.2± 0.9	0.362	0.148148
HOOSp7	3.0 ± 0.8	3.1 ± 0.8	0.432	0.155556
UCLA	4.4 ± 1.8	4.0 ± 1.6	0.458	0.162963
HHS	53.0 ± 11.9	56.6 ± 12.1	0.596	0.17037
HOOSp3	3.6 ± 0.6	3.6 ± 0.8	0.68	0.177778
BMI	32.4 ± 4.0	35.4 ± 5.0	0.871	0.185185
VTSF36	44.8 ± 9.6	46.2 ± 9.6	0.871	0.192593
HOOS Pain factor 2	0.02 ± 0.66	0.01 ± 1.11	0.931	0.2

Supplementary Table 9-3. Correlations between cytokine concentrations and hip pain

		HOOS													BPSF36
		Total	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**PC1**	**PC2**	
Fractalkine	**Corr.**	0.18	-0.063	-0.001	-0.217	-0.268	-0.096	-0.095	-0.032	-0.089	-0.158	-0.107	-0.19	0.089	-0.067
	P-value	0.212	0.664	0.994	0.131	0.06	0.506	0.51	0.825	0.54	0.275	0.461	0.185	0.54	0.646
IL10	**Corr.**	0.091	-0.047	-0.183	-0.193	-0.016	0.061	0.021	-0.22	-0.124	0.044	0.01	-0.085	-0.045	-0.099
	P-value	0.53	0.746	0.202	0.18	0.912	0.673	0.884	0.125	0.392	0.763	0.944	0.558	0.756	0.493
IL15	**Corr.**	0.009	-0.101	0.077	-0.102	-0.029	0.126	0.031	-0.073	-0.096	0.043	0.13	0.007	-0.019	-0.179
	P-value	0.953	0.484	0.595	0.483	0.841	0.384	0.832	0.615	0.506	0.765	0.37	0.96	0.896	0.214
IL6	**Corr.**	0.036	0.285*	0.061	-0.014	-0.031	0.201	-0.081	-0.178	-0.213	0.05	0.025	-0.006	-0.319*	-0.057
	P-value	0.804	0.045*	0.676	0.921	0.831	0.161	0.574	0.217	0.138	0.728	0.865	0.966	0.024*	0.696
MCP1	**Corr.**	0.028	-0.128	-0.078	0.042	-0.044	0.084	-0.025	-0.04	-0.046	-0.045	-0.016	-0.044	0.006	0.086
	P-value	0.848	0.374	0.589	0.771	0.762	0.564	0.862	0.782	0.75	0.754	0.911	0.763	0.967	0.553
TNFa	**Corr**	-0.039	0.083	0.202	0.003	-0.019	0.169	-0.071	-0.057	-0.008	0.137	0.022	0.033	-0.131	-0.146
	P-value	0.79	0.568	0.159	0.986	0.895	0.242	0.623	0.697	0.953	0.342	0.88	0.82	0.363	0.312
IP10	**Corr.**	-.294*	0.11	0.198	0.218	0.089	0.122	0.167	0.233	.390**	0.231	0.142	-0.223	0.11	0.198
	P-value	0.038*	0.447	0.168	0.128	0.54	0.397	0.246	0.103	.005**	0.106	0.326	0.12	0.447	0.168
MDC	**Corr.**	0.048	0.018	0.085	0.085	-0.077	0.109	0.03	-0.041	-0.071	0.005	0.064	-.302*	-0.048	0.018
	P-value	0.74	0.901	0.558	0.558	0.594	0.45	0.838	0.779	0.626	0.971	0.659	.033*	0.74	0.901

Abbreviations used in table: IL (interleukin), MCP (monocyte chemoattractant protein), MDC (macrophage derived chemokine), IP10 (interferon gamma-induced protein), TNF (tumor necrosis factor), PC (principle component), HOOS (Hip disability and osteoarthritis outcome score), SF36 (Short Form 36).

Supplementary Table 9-4. The Down and Black bias assessment checklist

			Petersson 1	Zhang 2015	Zhang 2014	Xin 2017	Wakitani 20	Van Spil 201	Sugiyama 2	Steinbeck 2	Poole 2016
REPORTING	Q1	Hypothesis/aim/objective clearly described	1	1	1	1	1	1	1	1	1
	Q2	Main outcomes in Introduction or Methods	1	1	1	1	1	1	1	1	1
	Q3	Patient characteristics clearly described	1	1	1	1	1	1	1	0	0
	~~Q4~~	~~Interventions of interest clearly described~~									
	Q5	Principal confounders clearly described	0	1	0	0	1	1	1	0	0
	Q6	Main findings clearly described	1	1	1	1	1	1	1	1	1
	Q7	Estimates of random variability provided for main outcomes	1	1	1	1	1	1	1	1	1
	~~Q8~~	~~All adverse events of intervention reported~~									
	Q9	Characteristics of patients lost to follow-up described	0					1			
	Q10	Probability values reported for main outcomes	1	1	1	1	1	1	1	1	1
EXTERNAL VALIDITY	Q11	Subjects asked to participate were representative of source population	1	1	1	1	0	1	1	0	0
	Q12	~~Subjects prepared to participate were representative of source population~~									
	Q13	~~Location and delivery of study treatment was representative of source population~~									
INTERNAL VALIDITY; BIAS; CONFOUNDING	Q14	~~Study participants blinded to treatment~~									
	Q15	~~Blinded outcome assessment~~									
	Q16	Any data dredging clearly described	1	1	1	1	1	1	1	1	1
	Q17	Analyses adjust for differing lengths of follow-up	0					1			
	Q18	Appropriate statistical tests performed	1	1	1	1	1	1	1	1	1
	Q19	~~Compliance with interventions was reliable~~									
	Q20	Outcome measures were reliable and valid	1	1	1	0	1	1	1	1	1
	Q21	All participants recruited from the same source population	1	1	1	1	1	1	1	1	1
	Q22	All participants recruited over the same time period	1	1	1	1	1	1	1	1	1
	Q23	~~Participants randomized to intervention(s)~~									
	Q24	~~Allocation of intervention concealed from investigators and participants~~									
	Q25	Adequate adjustment for confounding	0	0	0	0	0	1	1	0	1
	Q26	Losses to follow-up taken into account	0					0			
POWER	Q27	Sufficient power to detect clinically important effect at significance level of 0.05	0	0	0	0	0	1	0	0	0
Sum			12	13	12	11	12	17	14	10	11
Percentage			66.67%	86.67%	80.00%	73.33%	80.00%	94.44%	93.33%	66.67%	73.33%

			Marshall 200	Kosinska 20	Kobayashi 1	Jiao 2016	de Seny 201	Bassiouni 2(	Ahmed 2015	Vos 2013	Ling 2009
REPORTING	Q1	Hypothesis/aim/objective clearly described	1	1	1	1	1	1	1	1	1
	Q2	Main outcomes in Introduction or Methods	1	1	1	1	1	1	1	1	1
	Q3	Patient characteristics clearly described	1	1	1	1	1	0	1	1	1
	~~Q4~~	~~Interventions of interest clearly described~~									
	Q5	Principal confounders clearly described	1	1	0	1	1	0	1	0	1
	Q6	Main findings clearly described	1	1	1	1	1	1	1	1	1
	Q7	Estimates of random variability provided for main outcomes	1	1	1	1	1	1	1	1	1
	~~Q8~~	~~All adverse events of intervention reported~~									
	Q9	Characteristics of patients lost to follow-up described								0	1
	Q10	Probability values reported for main outcomes	1	1	1	1	1	1	1	1	1
EXTERNAL VALIDITY	Q11	Subjects asked to participate were representative of source population	1	1	1	1	1	1	1	1	1
	Q12	~~Subjects prepared to participate were representative of source population~~									
	Q13	~~Location and delivery of study treatment was representative of source population~~									
INTERNAL VALIDITY; BIAS; CONFOUNDING	Q14	~~Study participants blinded to treatment~~									
	Q15	~~Blinded outcome assessment~~									
	Q16	Any data dredging clearly described	1	1	1	1	0	1	1	1	1
	Q17	Analyses adjust for differing lengths of follow-up								1	1
	Q18	Appropriate statistical tests performed	1	1	1	1	1	1	1	1	1
	Q19	~~Compliance with interventions was reliable~~									
	Q20	Outcome measures were reliable and valid	1	1	1	1	1	0	1	1	1
	Q21	All participants recruited from the same source population	1	1	1	1	1	1	1	1	1
	Q22	All participants recruited over the same time period	1	1	1	1	1	1	1	1	1
	Q23	~~Participants randomized to intervention(s)~~									
	Q24	~~Allocation of intervention concealed from investigators and participants~~									
	Q25	Adequate adjustment for confounding	1	0	0	0	0	0	1	0	0
	Q26	Losses to follow-up taken into account								0	0
POWER	Q27	Sufficient power to detect clinically important effect at significance level of 0.05	1	0	1	0	1	0	0	0	1
Sum			15	13	13	13	13	10	14	13	16
Percentage			100.00%	86.67%	86.67%	86.67%	86.67%	66.67%	93.33%	72.22%	88.89%

			Wei 2014	Livshits 200	Kumm 2013	Chaganti 20	Sowers 199	Kelman 200	Gebhard 20	Cibere 2009
REPORTING	Q1	Hypothesis/aim/objective clearly described	1	1	1	1	1	1	1	1
	Q2	Main outcomes in Introduction or Methods	1	1	1	1	1	1	1	1
	Q3	Patient characteristics clearly described	1	1	0	1	1	1	1	1
	~~Q4~~	~~Interventions of interest clearly described~~								
	Q5	Principal confounders clearly described	1	1	1	1	1	1	0	1
	Q6	Main findings clearly described	1	1	1	1	1	1	1	1
	Q7	Estimates of random variability provided for main outcomes	1	1	1	1	1	1	1	1
	~~Q8~~	~~All adverse events of intervention reported~~								
	Q9	Characteristics of patients lost to follow-up described		1	1	0	1	0		
	Q10	Probability values reported for main outcomes	1	1	1	1	1	1	1	1
EXTERNAL VALIDITY	Q11	Subjects asked to participate were representative of source population	1	1	1	1	1	1	1	1
	Q12	~~Subjects prepared to participate were representative of source population~~								
	Q13	~~Location and delivery of study treatment was representative of source population~~								
INTERNAL VALIDITY; BIAS; CONFOUNDING	Q14	~~Study participants blinded to treatment~~								
	Q15	~~Blinded outcome assessment~~								
	Q16	Any data dredging clearly described	1	1	1	1	1	1	1	0
	Q17	Analyses adjust for differing lengths of follow-up		1	1	0	1	0		
	Q18	Appropriate statistical tests performed	1	1	1	1	1	1	1	1
	Q19	~~Compliance with interventions was reliable~~								
	Q20	Outcome measures were reliable and valid	1	1	1	1	1	1	1	1
	Q21	All participants recruited from the same source population	1	1	1	1	1	1	1	
	Q22	All participants recruited over the same time period	1	1	1	1	1	1	1	1
	Q23	~~Participants randomized to intervention(s)~~								
	Q24	~~Allocation of intervention concealed from investigators and participants~~								
	Q25	Adequate adjustment for confounding	0	1	1	1	1	1	0	0
	Q26	Losses to follow-up taken into account		0	1	0	0	0		
POWER	Q27	Sufficient power to detect clinically important effect at significance level of 0.05	0	1	1	1	0	1	0	1
Sum			13	17	17	15	16	15	12	12
Percentage			86.67%	94.44%	94.44%	83.33%	88.89%	83.33%	80.00%	80.00%

Supplementary Table 9-5. Correlation between cytokines and pain scores in rat model

			CCL22	EPO	IL13	IL10	IL18	IL1a	IL2	MCSF
ALL	Mean, SD		2376, 870.8	1480.9, 426.3	1572.1, 370.2	1800.2, 378	3089.4, 1258.8	2227.2, 689.8	2386.2, 1029.3	4406.4, 705.8
	Spearsman Correlation with Pain Score	r	.255*	-0.144	-.327**	-.282*	-0.036	-.405**	-0.147	-.482**
		p-value	0.045	0.259	0.009	0.025	0.782	0.001	0.251	0.000
Day 1	Mean, SD		1178.9, 139.3	1479.2, 359.7	1290.9, 289	1538.2, 312.4	3696.2, 700	3116.6, 947.8	2062, 718.4	5039.3, 447.4
	Spearsman Correlation with Pain Score	r	0.519	-0.627	-0.390	-0.186	-.729*	-0.661	-0.136	-0.390
		p-value	0.176	0.071	0.300	0.631	0.026	0.053	0.728	0.300
Day 3	Mean, SD		1614.4, 188.7	1463.3, 550.2	1559.6, 506	1722.9, 373	3056.4, 998.1	2232.2, 524.7	2201.8, 665	4903.7, 558.5
	Spearsman Correlation with Pain Score	r	0.276	-0.417	-0.350	-0.517	-0.417	-0.367	-0.350	-.833**
		p-value	0.499	0.277	0.371	0.161	0.277	0.346	0.371	0.003
Day 5	Mean, SD		1975, 315.2	1642.2, 521.7	1658.7, 396.8	2020.4, 524.2	3504.5, 1831.5	2238.5, 805.7	3066.7, 2202.6	4946.6, 677.6
	Spearsman Correlation with Pain Score	r	-0.318	0.452	-0.636	-0.134	0.544	-0.226	-0.126	-0.485
		p-value	0.466	0.233	0.066	0.741	0.135	0.573	0.757	0.194
Day 10	Mean, SD		2531.7, 564.3	1514.1, 491.4	1658.2, 179	1863.1, 217.2	2980.4, 1184.9	2034.6, 427.9	2534.2, 673.7	4096.7, 548.5
	Spearsman Correlation with Pain Score	r	0.558	-0.100	-0.617	-0.583	0.167	-0.100	-0.300	-.667*
		p-value	0.161	0.806	0.078	0.102	0.680	0.806	0.448	0.049
Day 14	Mean, SD		2909.6, 717.3	1225.7, 414.9	1371.9, 408.7	1604.8, 443.5	2458.4, 845	1749.8, 454.2	2016.8, 825.2	3779.8, 625.9
	Spearsman Correlation with Pain Score	r	0.078	0.017	-0.250	-0.167	0.300	-0.350	-0.167	-0.217
		p-value	0.859	0.967	0.532	0.680	0.448	0.371	0.680	0.590
Day 20	Mean, SD		3286, 492	1587.1, 327.2	1687.4, 346.4	1901.3, 284.2	3322.7, 1790.4	2121.3, 377.1	2403.6, 482	4151.6, 448.9
	Spearsman Correlation with Pain Score	r	-0.080	0.286	-0.580	-0.412	-0.017	-0.235	-0.345	-0.345
		p-value	0.869	0.472	0.105	0.284	0.967	0.557	0.379	0.379
Day 24	Mean, SD		3006.5, 727.7	1455, 256.8	1778.1, 179.9	1950.3, 219.9	2607.2, 850.7	2097.7, 337.9	2417.9, 419.7	3926.9, 238.9
	Spearsman Correlation with Pain Score	r	-0.066	0.135	-0.068	-0.042	0.354	-0.312	-0.042	-0.008
		p-value	0.872	0.739	0.868	0.918	0.364	0.429	0.918	0.984

			IL1b	IL4	IFNg	MIP3a	GMCSF	IL7	TNFa	VEGF
ALL	Mean, SD		1672.1, 391.1	1567.9, 407	1270.5, 428.4	1872.1, 478.3	1612.1, 373.2	3706, 1089.8	827, 284.3	2224.5, 370.2
	Spearsman Correlation with Pain Score	r	-.305*	-0.222	-.322*	-.328**	-.359**	-0.231	-0.212	-.479**
		p-value	0.015	0.080	0.010	0.009	0.004	0.069	0.096	0.000
Day 1	Mean, SD		1397.9, 325	1224.3, 343.1	967.1, 355.2	1619.2, 379.1	1327.7, 325.1	3925.3, 1279	589, 243.3	2201.4, 315.6
	Spearsman Correlation with Pain Score	r	-0.458	-0.390	-0.373	-0.441	-0.390	-0.237	-0.254	-0.475
		p-value	0.215	0.300	0.323	0.235	0.300	0.539	0.509	0.197
Day 3	Mean, SD		1682.6, 535	1500.1, 441.2	1283.3, 436.6	1832.2, 519.5	1610, 497.8	3962.1, 1323.4	819.5, 310.2	2363.2, 468.9
	Spearsman Correlation with Pain Score	r	-0.300	-0.383	-0.283	-0.517	-0.360	-0.150	-0.200	-0.483
		p-value	0.448	0.322	0.476	0.161	0.356	0.711	0.619	0.196
Day 5	Mean, SD		1749.3, 424.5	1799.1, 546.1	1445.1, 445.7	2206.2, 717.8	1678.4, 375.7	3727.2, 891.3	892.9, 311.6	2226.4, 446.4
	Spearsman Correlation with Pain Score	r	-0.636	-0.310	-.770*	-0.209	-0.552	-0.075	-.669*	-0.636
		p-value	0.066	0.433	0.012	0.603	0.128	0.853	0.047	0.066
Day 10	Mean, SD		1740.8, 185.1	1647.4, 182.1	1263.9, 362.8	1921.6, 310.5	1704.2, 179.4	3979.4, 1202.9	835.6, 173.9	2214.9, 215.3
	Spearsman Correlation with Pain Score	r	-0.583	-0.383	-0.433	-0.500	-0.617	-0.033	-0.050	-0.650
		p-value	0.102	0.322	0.256	0.178	0.078	0.935	0.902	0.058
Day 14	Mean, SD		1463.4, 426.7	1412.4, 453.6	1140.4, 499.8	1562, 474.1	1407.1, 405.1	2984.4, 744	750.7, 301.3	1925.3, 420.1
	Spearsman Correlation with Pain Score	r	-0.250	-0.250	-0.583	-0.167	-0.383	-0.217	-0.433	-0.350
		p-value	0.532	0.532	0.102	0.680	0.322	0.590	0.256	0.371
Day 20	Mean, SD		1793.7, 395.4	1649.6, 331.9	1365.2, 485.4	1950.1, 355.6	1758.8, 374	3632.8, 1147.4	924.8, 344.1	2289.8, 388
	Spearsman Correlation with Pain Score	r	-0.462	-0.345	-0.319	-0.555	-0.571	-0.202	-0.134	-0.462
		p-value	0.221	0.379	0.418	0.126	0.112	0.616	0.740	0.221
Day 24	Mean, SD		1877.3, 175.5	1742.3, 223.9	1428.5, 307	2013.2, 239.4	1798.5, 180.2	3730.7, 949.4	976.5, 152	2350, 156.8
	Spearsman Correlation with Pain Score	r	-0.076	-0.084	-0.101	-0.354	-0.346	0.034	0.228	-0.152
		p-value	0.852	0.836	0.803	0.364	0.377	0.934	0.570	0.708

			MCP1	IL5	GCSF	RANTES	IL6	GRO	IL17a	IL12p70
ALL	Mean, SD		4285.1, 864.7	1814.1, 359.1	1274.3, 390	7884.2, 2095.3	1196.6, 428.2	5480.9, 1427.4	2163.1, 512.6	1813.2, 426.6
	Spearsman Correlation with Pain Score	r	-.438**	-.377**	-.303*	-.370**	-.311*	-0.247	-.352**	-.362**
		p-value	0.000	0.002	0.016	0.003	0.013	0.051	0.005	0.004
Day 1	Mean, SD		4744.6, 679.1	1559.6, 365.4	980.1, 346.7	11585.7, 1941.9	921.7, 329.5	6599.9, 1571.7	1976.7, 483.3	1484.6, 387.5
	Spearsman Correlation with Pain Score	r	0.102	-0.509	-0.424	-0.356	-0.322	-0.220	-0.542	-0.424
		p-value	0.795	0.162	0.256	0.347	0.398	0.569	0.131	0.256
Day 3	Mean, SD		4339.5, 464.9	1767.4, 417.1	1220.2, 430.4	9226.8, 1066.4	1216.9, 455.6	5658.5, 1462.4	2182.8, 567.3	1833.8, 602.2
	Spearsman Correlation with Pain Score	r	-.767*	-0.517	-0.317	-0.400	-0.167	-0.267	-0.517	-0.450
		p-value	0.013	0.161	0.422	0.299	0.680	0.503	0.161	0.235
Day 5	Mean, SD		5164.2, 1216.7	1935.6, 335.1	1451.9, 365	7826.4, 764.1	1350.5, 463	5527.8, 1423.1	2428.4, 472.6	1918.1, 435.4
	Spearsman Correlation with Pain Score	r	-0.611	-0.494	-.676*	-0.611	-.770*	0.042	-0.084	-0.561
		p-value	0.082	0.185	0.044	0.082	0.012	0.918	0.837	0.121
Day 10	Mean, SD		3948.4, 731.6	1890.4, 190.5	1318.7, 298	7106.1, 824.9	1183.5, 381.6	5609.5, 1440.6	2144, 474.3	1937.8, 230.2
	Spearsman Correlation with Pain Score	r	-0.317	-0.567	-0.450	-0.467	-0.383	0.017	-.683*	-0.617
		p-value	0.422	0.115	0.235	0.215	0.322	0.967	0.041	0.078
Day 14	Mean, SD		3741.4, 850.3	1605.4, 423.3	1140.1, 447.8	6665.5, 1535.4	1076, 488.8	4401.2, 805.1	1828, 642.6	1610.4, 462
	Spearsman Correlation with Pain Score	r	-0.200	-0.250	-0.583	-0.300	-.683*	-0.200	-0.083	-0.200
		p-value	0.619	0.532	0.102	0.448	0.041	0.619	0.838	0.619
Day 20	Mean, SD		4008.5, 668.9	1925, 353.8	1359.1, 433.6	6450.3, 684.9	1275, 499.1	5387.1, 1476.4	2249.8, 547.3	1927.7, 380.2
	Spearsman Correlation with Pain Score	r	-0.387	-0.639	-0.303	-0.067	-0.319	-0.151	-0.328	-0.529
		p-value	0.318	0.064	0.444	0.869	0.418	0.709	0.405	0.149
Day 24	Mean, SD		4048.8, 460.4	2015.2, 179.7	1449.8, 248.3	6328.9, 438.7	1352.9, 305.9	5182.4, 1169.1	2331.8, 185.6	1980, 229.7
	Spearsman Correlation with Pain Score	r	-0.228	-0.245	-0.228	-0.498	-0.101	-0.160	0.354	-0.549
		p-value	0.570	0.541	0.570	0.181	0.803	0.692	0.364	0.131

Supplementary Table 9-6. The OARSI OA scores and IF findings of all joints

Week	Animal	CCL22 staining	Matrix Loss Width (um)			Degeneration Score				Total degeneration width (um)	Significant degeneration width (um)	Osteophytes	Synovial inflammation
			Surface 0%	Mid depth 50%	Tidemark 100%	Medial Zone 1	Central Zone 2	Lateral Zone 3	Total				
1	1	No	0	0	0	0	0	0	0	0	0	0	0
	2	Yes	453	175	43	1	0	0	1	869	538	0	0
	3	Yes	0	0	0	0	0	0	0	0	0	0	0
	1wk mean	66.67%	151.00	58.33	14.33	0.33	0.00	0.00	0.33	289.67	179.33	0.00	0.00
2	4	No	0	0	0	0	0	0	0	0	0	0	0
	5	Yes	435	0	0	3	5	5	14	2374	1126	0	4
	6	No	0	0	0	0	0	0	0	0	0	0	0
	2wk mean	33.33%	145.00	0.00	0.00	1.00	1.67	1.67	4.67	791.33	375.33	0.00	1.33
3	7	Yes	0	0	0	0	0	0	0	0	0	0	0
	8	Yes	0	0	0	0	0	3	3	670	391	0	0
	9	Yes	0	0	0	0	0	0	0	0	0	0	0
	3wk mean	100%	0.00	0.00	0.00	0.00	0.00	1.00	1.00	223.33	130.33	0.00	0.00
4	10	Yes	1056	416	0	0	2	3	5	1108	611	4	4
	11	Yes	924	508	0	0	5	5	10	1473	465	4	4
	12	Yes	986	265	0	0	5	4	9	1279	596	4	4
	13	Yes	932	366	0	0	2	5	7	1286	318	4	4
	14	Yes	560	0	0	0	4	5	9	807	0	3	3
	15	Yes	773	158	0	0	3	5	8	1233	140	4	4
	16	Yes	777	0	0	0	2	5	7	965	149	4	4
	17	Yes	1034	459	0	2	4	5	11	1438	678	4	4
	18	Yes	847	337	0	0	3	4	7	1209	330	4	4
	4wk mean	100%	876.56	278.78	0.00	0.22	3.33	4.56	8.11	1199.78	365.22	3.89	3.89

Supplementary Table 9-7 Correlation between CCL22 and OARSI scores

		Matrix Loss Width (um)			**Degeneration Score**				**Total degeneration width (um)**	**Significant degeneration width (um)**	**Osteophytes**	**Synovial inflammation**
		Surface 0%	Mid depth 50%	Tidemark 100%	Medial Zone 1	Central Zone 2	Lateral Zone 3	Total				
Spearman Correlation with OARSI Score	**r**	.786*	0.587	N/A	0.577	0.334	-0.483	0.337	0.476	.905**	0.577	0.577
	p-value	0.021	0.126	N/A	0.134	0.419	0.225	0.414	0.233	0.002	0.134	0.134

Chapter Ten: Appendix

10.1 A Prospective Study of Serum Cytokine Profile in Individuals at Risk of Post-traumatic Osteoarthritis (324)

Authors: Guomin Ren, Jackie L. Whittaker, Carolyn. A. Emery, Roman J. Krawetz

Purpose

There is increasing evidence that low-level chronic inflammation plays a pivotal role in the onset and pathogenesis of osteoarthritis (OA). In previous studies, we successfully utilized cytokine association networks to identify a conserved signature for MRI-defined OA in individuals at risk of post-traumatic OA. However, the value of MRI-defined OA as an early OA diagnostic remains under debate. Therefore, the purpose of this study was to: 1) compare serum cytokine profiles in participants at risk of OA due to a youth sport-related intra-articular knee injury 3-10 years previously (with and without MRI-defined OA; MRI+ and MRI-) with healthy controls and individuals clinically diagnosed with knee OA; and 2) determine if participants at risk of OA with MRI-defined OA at baseline demonstrate inflammatory profiles more similar to participants with a clinical diagnosis of OA with successive annual follow-ups.

Methods

Participants:

Risk of OA: A sub-sample (n=24) of the Alberta PrE-OA (Youth Prevention of Early Osteoarthritis) cohort that had completed baseline MRI studies and 3 years (baseline,

year 1 and year 2) serum cytokine profiling. This included 10 MRI + and 14 MRI - participants.

Control: Healthy volunteers (n=30) recruited at the Human Performance Lab at the University of Calgary.

OA: Individuals with a clinical diagnosis (Sport and Exercise Medicine Physician) of knee OA (n=30).

Procedures:

At baseline individuals at risk of OA underwent bilateral routine clinical MRI (axial, coronal and sagittal proton density and proton density fat saturation sequences; 1.5 Tesla) studies. MRI defined-OA was based on established criteria and derived from MRI OA Knee Scores. Inflammatory profiles (41 cytokines) from serum samples collected at baseline, year 1 and year 2 were analyzed using the Human Cytokine Panel (Millipore) on the Luminex platform.

Data Analysis

One-way ANOVA, Principle component analysis (PCA) and visualization were performed using SPSS 24.0 (SPSS, Inc., Chicago IL) with $\alpha=0.001$ ($\alpha=0.05/41$ comparisons≈ 0.001). Cytokine networks for Risk of OA (3-time points), Control and OA groups were created and analyzed using R and minet package as previously described. Briefly, for all cytokine pairs, the pairwise mutual information, which was considered as cytokine interaction strength, were calculated. Then, by applying the data

processing inequality, most indirect interactions (lowest strength, or smallest MI value of any 3-cytokine loops) were removed. Finally, weighted networks were constructed with cytokines as nodes and remaining interactions as connecting lines. Network connectivity was defined as the average betweenness of all cytokines in the network.

Results

The mean age of Risk of OA, Control and OA participants were 22.6 ± 2.3, 40.0 ± 9.5, 60.4 ± 10, respectively. Nine individual cytokines (EGF, Eotaxin-1, GROa, CCL22, IP10, CCL2, MIP1a, MIP1b, RANTES, VEGFa) and overall cytokine profiles were significantly different across groups (Risk of OA, Control and OA). These differences could be easily visualized by plotting 3 components from PCA (**Figure 10-1**). Although overlap existed, and there was no difference between the MRI+ and MRI-, the changes between annual testing were statistically significant within the Risk of OA group. With that said, this group was slowly departing from, rather than moving towards, OA (regardless of MRI-defined OA status; **Figure 10-2a, b**). A similar result was found in cytokine association networks in terms of the network connectivity. The connectivity of Risk of OA (MRI+ and MRI- together) networks decreased at year 1 and year 2, increasing the gap between the Risk of OA and OA groups (**Figure 10-2**c). The OA network had the highest connectivity, indicating that cytokines in the OA group were more highly correlated with each other than the other groups. This is consistent with our previous studies.

Conclusions

We have identified that individuals at risk of OA have a unique cytokine profile, which is distinct from controls and individuals with a clinical diagnosis of OA. Although the cytokine profile of the MRI+ and MRI- changed over time, it was not in the direction of either the control or clinically diagnosed OA groups. One possible explanation is that another covariate (i.e., age, sex) is influencing serum cytokine profile. Future studies with a larger sample size that enable multivariable analyses should be undertaken.

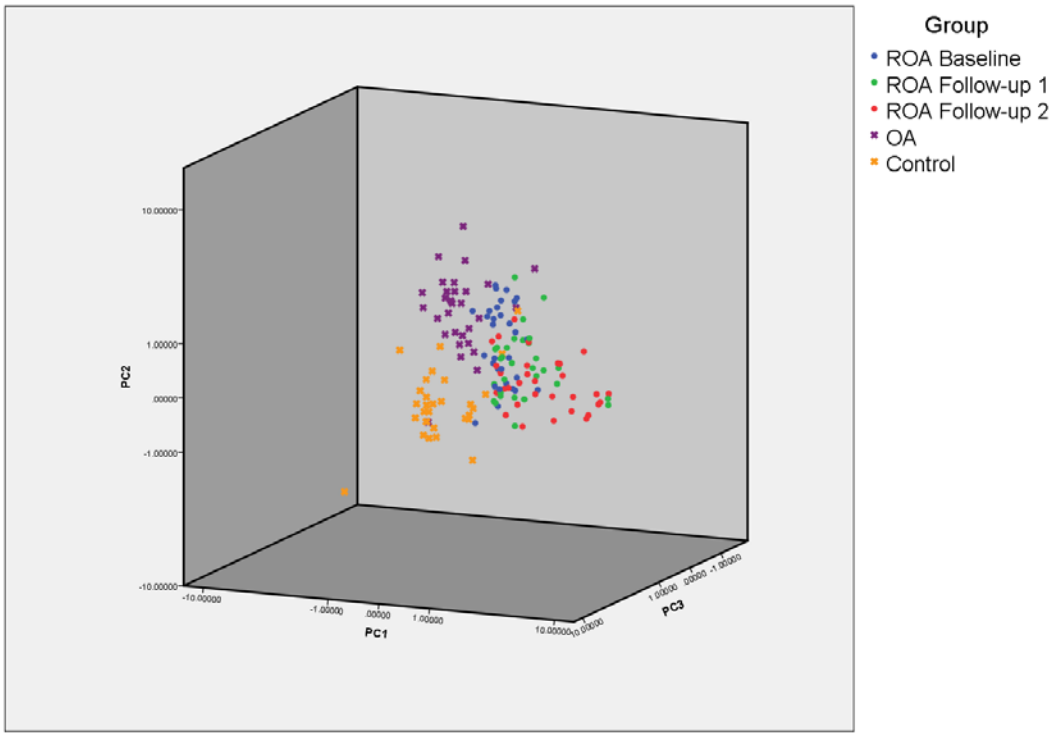


Figure 10-1. Scatter plot of 3 principle components of all groups

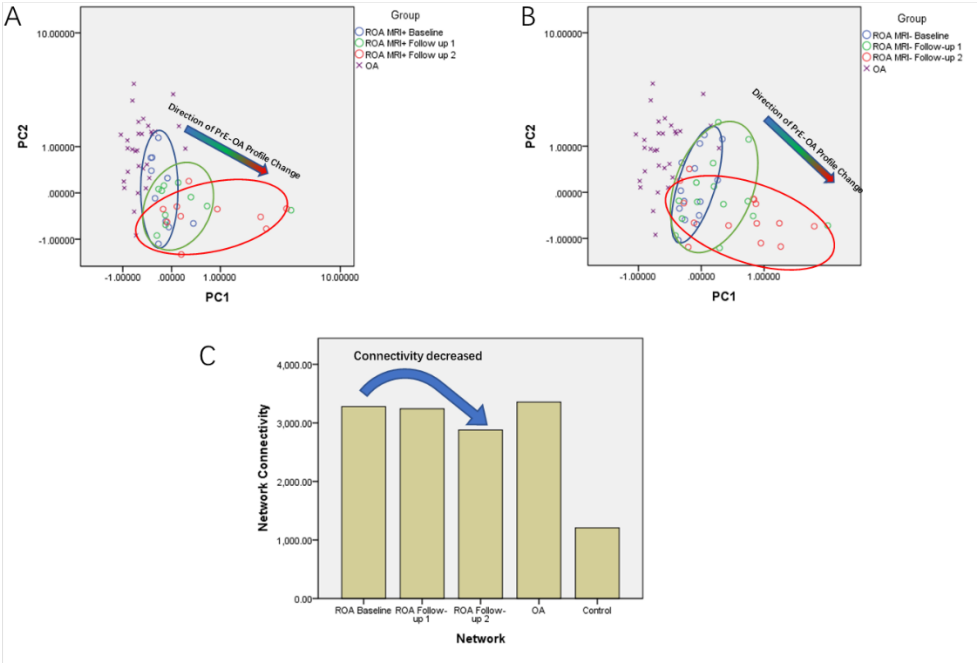


Figure 10-2. Scatter plot of At Risk (MRI+ only) and OA. B. Scatter plot of PrE-OA (MRI- only) and OA. C. Connectivity of networks for At Risk (3 time points separately) OA and control

10.2 Network Analysis of Cytokine Profiles Reveals Potential Biomarkers for MRI-defined OA

Authors: Guomin Ren, Jackie L. Whittaker, Carolyn. A. Emery, Roman J. Krawetz

Purpose

Previous studies have shown that inflammatory mediators such as cytokines might be potential biomarkers for detecting the onset of osteoarthritis (OA). However, classical statistical methods (e.g. compare means of variables) might not be sufficient to interrogate the complexity (e.g. redundancy, routes of feedback and cross-talk) of cytokine signaling in the pathogenesis of OA. Network-based approaches have recently emerged as one of the powerful systems biology tools for biomarker discovery studies and understanding complex biological systems. The aims of this study were: 1. To construct two cytokine interaction networks for participants with and without MRI-defined knee OA. 2. To compare two networks and identify the most different cytokine(s) between two networks.

Methods

Data acquirement

Fifty participants who sustained a youth sport-related intra-articular knee joint injury and 50 non-injured controls with sex and sport matched to the knee injury cohort were included in this study. Cytokine profiles in serum samples from the cohorts were analyzed using the Human Cytokine Panel (Millipore) on the Luminex platform. The following cytokines were examined by Luminex in this study: EGF, Eotaxin, FGF2,

Flt3L, Fractalkine, GCSF, GMCSF, GROα, IFNα2, IFNγ, IL1α, IL1β, IL1rα, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12 (p40), IL12 (p70), IL13, IL15, IL17A, IL18, IP10, MCP1, MCP3, MDC, MIP1α, MIP1β, PDGFAA, PDGFAB/BB, RANTES, sCD40L, TGFα, TNFα, TNFβ, VEGFA. MRI OA features including bone marrow lesion (BML), cartilage, meniscus, ligament and synovitis in both injured and non-injured knees were evaluated using MRI OA scores (MOAKS). MRI-defined OA was based on established criteria using MOAKS.

Network Construction

Two networks were constructed independently from cytokine profiles of participants with and without MRI-defined OA followed the following steps. First, the pairwise mutual information (MI) were estimated for all cytokine pairs. MI is a measure of the mutual dependence between two variables similar to Pearson Correlation but more robust to manifest dependency. In this study, MI is considered as the strength of pairwise cytokine interaction. Second, by applying the data processing inequality (an information theoretic concept), most indirect interactions (lowest strength, or smallest MI value of any 3-gene loops) were removed. Finally, weighted networks were constructed with cytokines as nodes and remaining interactions as edges. ARACNe software was used for network construction.

Network Analysis

To quantify the topology of two networks, 6 node parameters (node betweenness, bridging, degree, eigenvector, radiality, stress) of both networks were computed using CentiScaPe plugin implemented in Cytoscape 3 software. To identify the most different

cytokines between 2 networks, the dynamic rewiring score (Dn-score), which is the standardized Euclidean Distance of node vectors in two networks was introduced to measure the "topological change" of each cytokine between 2 networks. Dn-scores were calculated using DyNet plugin implemented in Cytoscape 3 software.

Results

The topology of two networks were visually different (**Figure 10-3**). The average distance (interaction) of OA network (0.00697) was lower than that of non-OA network (0.00755). For most cytokines, different patterns were present in all 6 parameters between two networks (**Figure 10-4**). The top 5 different cytokines were identified as follows: GROα, CCL22, Fractalkine, IP10 and PDGFBB based on degree corrected Dn-score of each cytokine.

Conclusions

We have constructed cytokine networks for participants with and without MRI-defined OA. The two networks presented different topologies even though the levels of each cytokine expression were relatively indiscriminate between the two groups. The results indicated that differences existed in cytokine interactions between two groups and the network-based analysis approach we use was sufficiently sensitive to detect these differences. Moreover, the OA network had a lower average distance than the non-OA network. This suggested that cytokine interactions/associations are stronger in MRI-OA participants than those in non-MRI-OA. We also identified top 5 different cytokines

between two networks. The results were consisted with our previous studies in which both CCL22 and IP10 were found correlated with OA pain and structural changes within the joint. Overall, we have developed a new method to investigate cytokine profiles for the onset of OA and identified potential biomarkers for MRI-defined OA.

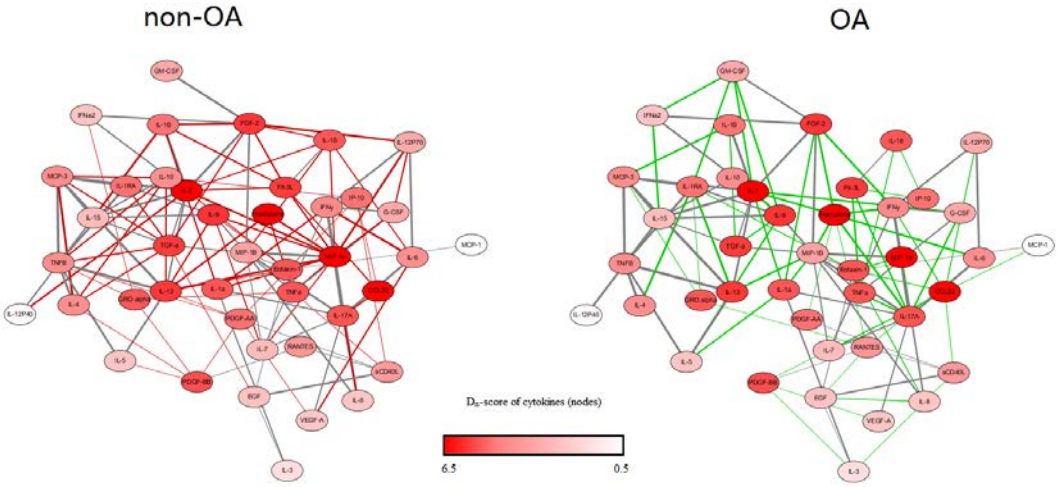


Figure 10-3. Cytokine networks of OA and non-OA group. Green edges: Interactions only exist in OA. Red edges: Interactions only exist in non-OA network. Grey edges: Interactions exist in both networks.

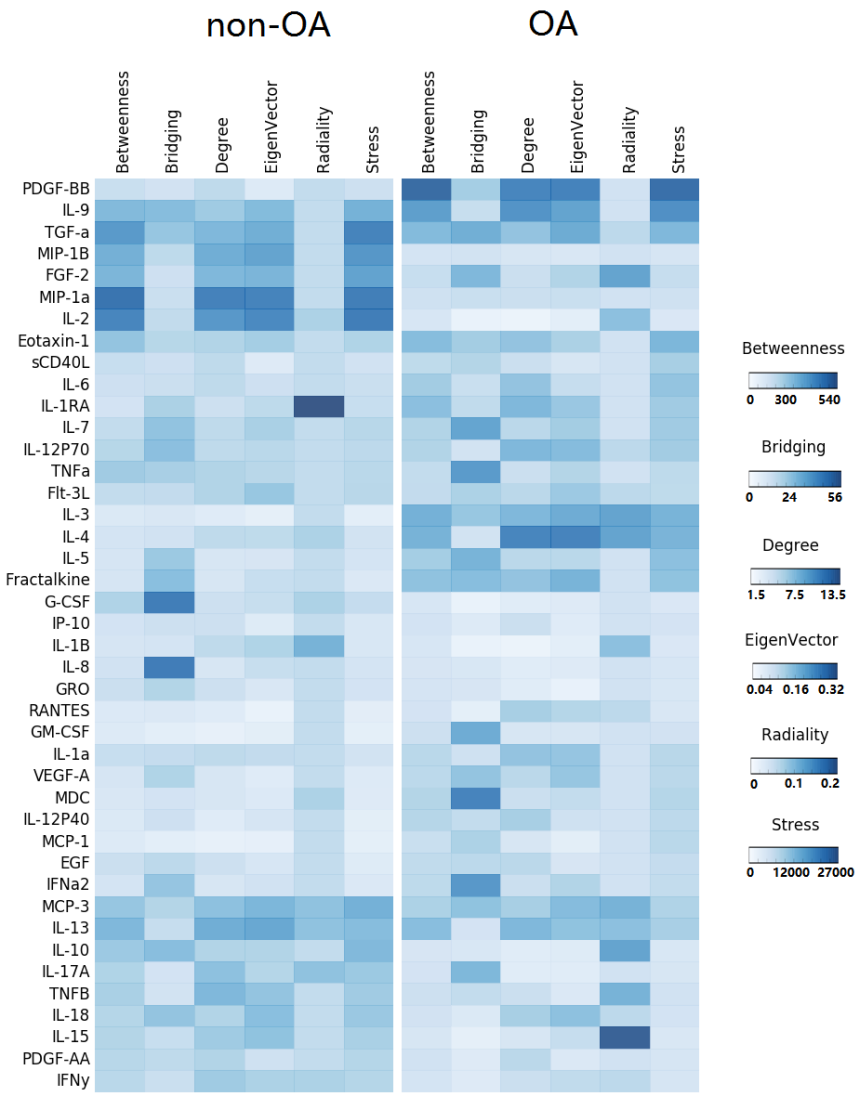


Figure 10-4. Heatmap of parameters of two networks. Network parameters are different between participants with vs without MRI-defined.

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