Can analysis of pro- and anti-inflammatory cytokines in matched synovial fluid and serum determine the inflammatory state in knee osteoarthritis?

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Abstract

Background: The concentrations of pro- and anti-inflammatory cytokines in biological specimens may help to determine the inflammatory state of knee osteoarthritis (KOA) in patients. Matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) are produced in osteoarthritis (OA) and rheumatoid arthritis (RA). Pro-inflammatory cytokines initiate a sequence of events which directly leads to joint destruction, through the subsequent production of matrix metalloproteinases (MMPs) that degrade bone.

Objectives: To determine the concentration of IL-6, TNF-α, IFN-γ, IL-17A, IL-13 and IL-10 in the serum and synovial fluid (SF) samples from KOA patients and healthy (asymptomatic) individuals. To compare total MMP-9 concentrations in plasma samples from KOA patients versus the total MMP-9 concentration in the healthy volunteer group. To interpret results to determine the inflammatory state of KOA in patients.

Methods: A panel of six cytokines (pro- and anti-inflammatory) were quantified using matched serum and SF from KOA patients undergoing total knee replacement (TKR) surgery, while only serum was used for cytokine analysis in the healthy group due to unavailability of SF in healthy individuals. There were two groups in the study: a group of healthy volunteers recruited from the Institute of Technology Carlow; and a group of KOA patients undergoing TKR surgery at AutEven Hospital, Kilkenny. The enzyme-linked immunosorbent assay (ELISA) was used to quantify cytokines (IL-6, TNF-α, IFN-γ, IL-10, IL-13 and IL-17A) in serum and SF of individual patients and volunteers in the study. For total MMP-9 concentration comparison in KOA and healthy plasma samples, gelatin zymography was carried out followed by band density measurements using ImageJ software analysis.

Results: Two cytokines measured, IL-6 and IL-10, showed significant difference in serum between the disease and healthy group in the study. Two cytokines measured, IFN-γ and IL-13, were high in the serum of healthy volunteers in the study. In individual KOA patients, total MMP-9 mean concentrations in plasma were higher than in the healthy samples with a significant difference of 0.03 ($P<0.05$).

Conclusion: Analysis of matched SF and serum confirmed local cytokine secretion within the joint of KOA patients. The concentrations of cytokines in serum were low in many cases which indicated KOA with low inflammation, while other patients had high concentrations of pro-inflammatory cytokines which indicated inflammatory OA. High risk candidates for autoimmune disease were identified in the healthy volunteer group. This may have indicated that the analysis of cytokines might be used as a screening tool for the early detection of autoimmune disease.

List of abbreviations

- ADAMS: disintegrin and metalloproteinases.
- ADAMTS: a disintegrin and metalloproteinase with thrombospondin-1 domains.
- APS: ammonium persulfate.
- AS: ankylosing spondylitis.
- Bromo blue: bromophenol blue.
- $CaCl_2$: calcium chloride dihydrate.
- catalogue #: catalogue number.
- CR: cruciate retaining.
- CRP: C-reactive protein.
- CV: coefficient of variance.
- DAMPs: damage-associated molecular patterns.
- diH_2O: sterile deionised water.
- DMARDs: disease-modifying anti-rheumatic drugs.
- DMOADs: disease modification osteoarthritis drugs.
- DPI: dots per inch.
- ECM: extracellular matrix.
- EDTA: ethylenediaminetetraacetic acid.
- ELISA: enzyme linked immunosorbent assay.
- ESR: erythrocyte sedimentation rate.
- FDA: U.S. food and drug administration.
- H_2O: water.
- HCl: hydrochloric acid.
- IA: inflammatory arthritis.
- IBS: irritable bowel syndrome.
- IFN-γ: interferon-gamma.
- IL: interleukin.
- JIA: juvenile idiopathic arthritis.
- JSN: joint space narrowing.
- KL: Kellgren and Lawrence.
- KOA: knee osteoarthritis.

- LC-MS/MS: liquid chromatography tandem-mass spectroscopy.
- mean ± SD: mean ± standard deviation.
- MHC: major histocompatibility complex.
- MMP: matrix metalloproteinase.
- $mol.L^{-1}$: moles.
- MRM: multiple reaction monitoring.
- mRNA: messenger ribonucleic acid.
- MS: mass spectroscopy.
- *n*: number of subjects.
- NaCl: sodium chloride.
- NaN_3: sodium azide.
- NGAL: neutrophil gelatinase-associated lipocalin.
- NIH: national institutes of health.
- NSAIDs: non-steroidal anti-inflammatory drugs.
- OA: osteoarthritis.
- PA: plasminogen activator.
- PCL: posterior cruciate ligament.
- $pg.mL^{-1}$: picograms per millilitre.
- PS: posterior.
- PSA: psoriatic arthritis.
- *P*-value: statistical significance.
- *q*PCR: reverse transcriptase polymerase chain reaction.
- RA: rheumatoid arthritis.
- rpm: revolutions per minute.
- RT: ambient room temperature.
- SDS: sodium dodecyl sulfate.
- Streptavidin-HRP: streptavidin-horseradish peroxidase.
- Synovial fluid: SF.
- TEMED: N,N,N', N'-Tetramethylethylenediamine.
- TGF-β: transforming growth factor-beta.
- Th17 cells: T-helper cells.
- TIMP: tissue inhibitor of matrix metalloproteinase.

- TKR: total knee replacement.
- TLR: toll-like receptors.
- TMB: 3,3',5,5'-Tetramethylbenzidine.
- TNF-α: tumour necrosis factor-alpha.
- TRIS: trisaminomethane.
- *U*: "Mann-Whitney *U"* value.
- VEGF: vascular endothelial growth factor.
- WB MMP-2: Bio-Techne western blot MMP-2 standard.
- WB MMP-9: Bio-Techne western blot MMP-9 standard.
- *z*: *z* score which is the "standardised test statistic" value.

List of materials and equipment for ELISA assays

- VWR CompactStar CS4 benchtop centrifuge (with angle rotor), catalogue number (catalogue #): 521-2854.
- Collection tubes for synovial fluid (SF): Greiner VACUETTE® Z no additive tubes, 4mL, white/black, 13x75, ridged, catalogue #: 454001.
- Collection tubes for plasma: Greiner VACUETTE® 3mL 9NC sandwich tube with 3.2% sodium citrate, ridged, screw cap premium, catalogue #: 454325
- Collection tubes for serum: Greiner VACUETTE® Z serum sep clot activator, 4mL, Gold/Gold, 13x75, ridged, catalogue #: 454071.
- VersaMax microplate reader from Molecular Devices with the ELISA plate reading and data analysis program, SoftMax Pro 6.2.2.
- R&D Systems precoated IL-6 ELISA kit with sufficient reagents for testing, catalogue #: D6050.
- 2X Affymetrix eBioscience human IL-6 platinum precoated ELISA kits with sufficient reagents for testing, catalogue #: BMS213/2CE.
- 2X Affymetrix eBioscience human IFN-γ platinum precoated ELISA kits with sufficient reagents for testing, catalogue #: BMS228.
- 2X Affymetrix eBioscience human IL-13 platinum precoated ELISA kits with sufficient reagents for testing, catalogue #: BMS231/3.
- Biolegend human IL-10 ELISA MAX Deluxe kit consisting of 5 uncoated ELISA plates with sufficient reagents for testing, catalogue #: 430604.
- Biolegend human TNF-α ELISA MAX Deluxe kit consisting of 5 uncoated ELISA plates with sufficient reagents for testing, catalogue #: 430204.
- Biolegend human IL-17A ELISA MAX Deluxe kit consisting of 5 uncoated ELISA plates with sufficient reagents for testing, catalogue #: 433914.
- Hanil BiMed Inc. Model Smart R17 Micro Refrigerated Centrifuge, supplied by Medical Supply Company, Radius of rotor: 71.2mm, Max speed: 17,000rpm.
- Labnet Orbit 300 laboratory shaker, speed range of 100 to 1,200rpm with a motion/orbit size which was 3mm circular, catalogue #: S2030-300-B.
- DAIHAN Scientific vortex mixer VM-10 model, supplied by Lennox, speed range of 0 to 3,300rpm in an orbital motion, catalogue #: EDHVM-10.

List of materials and equipment for gelatin zymography

- VWR CompactStar CS4 benchtop centrifuge (with angle rotor), catalogue: 521-2854.
- Collection tubes for plasma: Greiner VACUETTE® 3mL 9NC sandwich tube with 3.2% sodium citrate, ridged, screw cap premium, catalogue #: 454325
- Sigma Aldrich gelatin from bovine Skin (type B), catalogue #: 69382- 100g.
- Alfa Aesar bromophenol blue (bromo blue), catalogue #: AAA18469-18.
- Merck sodium azide, catalogue #: 1.06688.0100.100g.
- BioChemica Triton X-100, catalogue #: A1388.
- BioChemica glycine pure, catalogue #: A4554.
- BioChemica glycerol anhydrous pure, catalogue #: A2364.
- Scientific & Chemical Supplies sodium chloride, catalogue #: SO105.
- Sigma-Aldrich Trisaminomethane (TRIS), catalogue #: 252859.
- Sigma-Aldrich N,N′-Methylenebisacrylamide, catalogue #: M7256.
- Sigma-Aldrich Acrylamide, catalogue #: A8887.
- BioChemica calcium chloride 2-hydrate, catalogue #: A1873.
- BioChemica Coomassie® brilliant blue R250, catalogue #: A1092.
- BioChemica N,N,N',N'-Tetramethylethylenediamine (TEMED), catalogue #: A1148.
- J.T. Baker sodium dodecyl sulfate (SDS), catalogue #: 4095-02.
- BioChemica protein marker VI (10 to 245kDa) pre-stained protein ladder, catalogue #: A8889.
- Bio-Techne recombinant human MMP-2 western blot standard (WB MMP-2), catalogue #: WBC025
- Bio-Techne recombinant human MMP-9 western blot standard (WB MMP-9), catalogue #: WBC018.
- ATTO model AE6450 mini-slab size electrophoresis system. Distributed by Medical Supply Co., Ltd.
- BioRad PowerPac™ basic power supply 10 to 300V, catalogue #: 1645050.
- Hanna Instruments Model PH20-01 Easy-Use basic pH benchtop meter.
- StarLab 0.1-10µl round gel loading pipette tips, catalogue #: I1010-3000.

- Labnet Orbit 300 laboratory shaker, speed range of 100 to 1,200rpm with a motion/orbit size which was 3mm circular, catalogue #: S2030-300-B.
- Lennox vortex mixer VM-10 model, made by DAIHAN Scientific Co., Ltd., speed range of 0 to 3,300rpm in an orbital motion, catalogue #: EDHVM-10.

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Chapter 1: Literature Review

1.1 General introduction

Autoimmune conditions are inflammatory diseases involving specific organs where the immune system mistakenly attacks and destroys healthy body tissue (Ademowo *et al.*, 2013). There are over one hundred forms of arthritis in adults and children, of which many involve the inflammatory response (Ademowo *et al.*, 2013). Historically, osteoarthritis (OA) was not thought to be an inflammatory process, although research in the last decade has indicated it is (Bondeson, 2015; Ene *et al.*, 2015). Early detection of some forms of arthritis is ideal but remains challenging. Early treatment may even "switch off" symptoms of the condition (state of remission), ceasing irreversible joint destruction (Heidari, 2011). Conventionally, the development of drugs for inflammatory arthritis, especially rheumatoid arthritis (RA) has depended on extensive and time-consuming drug trials in order to determine if this treatment would benefit patients (Fleishaker, 2012). In order to determine the outcome of a drug therapy on patients in a shorter period, translational medicine has gained considerable interest and involves the use of biomarkers to interpret outcomes from clinical studies (Fleishaker, 2012).

The U.S. food and drug administration (FDA) and the national institutes of health (NIH) council, known as the FDA-NIH Working Group, established harmonised biomarker terminology. They defined a biomarker as "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions" (FDA-NIH Biomarker Working Group, 2016). Examples of biomarkers include molecular, histologic, radiographic, or physiologic characteristics. Biomarkers are classified into groups: susceptibility/ risk biomarkers; diagnostic biomarkers; monitoring biomarkers; predictive; pharmacodynamics response biomarkers; and safety biomarkers (FDA-NIH Biomarker Working Group, 2016). Biological biomarkers can be analysed from plasma, serum, synovial fluid (SF) and urine, ideally in a non-invasive manner so as to avoid patient complications. Qualitative biomarkers are specific to particular groups of sufferers, whereas quantitative biomarkers refer to those biomarkers where the concentration fluctuates in the patient group (Burska *et al.*, 2014).

Several techniques are employed for cytokine analysis in biological specimens. These include antibody and mass spectroscopy techniques. Each technique offers advantages and disadvantages that must be considered prior to testing (Ademowo *et al.*, 2013). The enzyme linked immunosorbent assay (ELISA) is described as the "gold standard" for quantifying cytokines (Burska *et al.*, 2014). The drawback of ELISA kits is only one cytokine can be analysed per kit, making multiple cytokine analysis very expensive (Burska *et al.*, 2014). The high dose hook effect is a problem encountered in immunoassays, causing false-negative results (Namburi *et al.*, 2014).When the concentration of a cytokine rises above a certain point, the system becomes saturated (in the case of the well on the ELISA plate) and loss of signal occurs whereby accurate quantification is not achieved. The concentration of the cytokine quantified may not be accurate as a result. To determine if the hook effect is present, samples are tested undiluted and using a dilution factor (Namburi *et al.*, 2014).

Excess manufacture of matrix metalloproteinases (MMPs) have been linked to tissue damage in inflammatory disorders including RA and OA (Snoek-van Beurden and Von den Hoff, 2005). MMPs are produced and released in the latent or inactive form, where they are referred to as a pro-MMP, proenzyme or zymogen (Snoek-van Beurden and Von den Hoff, 2005). Latent MMPs must be activated before extracellular matrix (ECM) cleavage can be achieved. Inhibitors are responsible for the regulation of the activity of MMPs. Tissue inhibitor of metalloproteinases (TIMPs) are the most important type of inhibitor in this process (Snoek-van Beurden and Von den Hoff, 2005). The balance of TIMPs and MMPs is the main factor causing degradation of ECM proteins (Snoek-van Beurden and Von den Hoff, 2005). Gelatin zymography is used for analysing MMP-2 and MMP-9 which offers greater sensitivity than other methods such as western blotting (Frankowski *et al.*, 2012). Advantages of gelatin zymography include the ability to identify active and latent forms of these MMPs, good quantitation and it is a relatively straightforward assay (Frankowski *et al.*, 2012).

1.2 Inflammatory forms of arthritis

1.2.1 Overview of inflammatory arthritis

Inflammatory arthritis (IA) refers to a broad range of autoimmune conditions involving inflammation of joints. It affects most joints, involving leukocyte infiltration of SF with systemic symptoms. It significantly worsens over-time leading to joint destruction and restricted movement causing disability in adults over fifty-five years old (Ademowo *et al.*, 2013). Healthcare challenges include: detection and diagnosis of early IA; determining those with a worse prognosis; limited medical resources where treatment can be expensive, for example disease-modifying anti-rheumatic drugs (DMARDs) are expensive; and finally selecting appropriate therapies (Ademowo *et al.*, 2013). IA has an enormous impact on the economy and with limited resources; a more feasible form of treatment must be developed (Ademowo *et al.*, 2013). IA types include RA, ankylosing spondylitis (AS), juvenile idiopathic arthritis (JIA), and psoriatic arthritis (PSA) (Barber *et al.*, 2016).

1.2.2 Pathophysiology of inflammatory arthritis

The pathogenic immune response is similar to the inflammatory response associated with joint destruction in IA. The synovium of the joint becomes inflamed which is referred to as "synovitis" (Minnock, 2012). The histological features in the pathology of IA include inflammatory synovitis and pannus formation (Scott *et al*., 2015). Inflammatory synovitis is seen in RA, with synovial hyperplasia and the influx of macrophage and fibroblast synovial cells T-cells, B cells and plasma cells infiltrate the sub-lining layer (Figure 1.1) (Scott *et al*., 2015). Pannus formation occurs (formation of synovial tissue) which contains large volumes of proteoglycans, causing the continued extension of the pannus through the cartilage (Scott *et al*., 2015). Late-stage RA is characterised by a fibrotic pannus with low vascularity (Scott *et al*., 2015). Acute inflammation occurs by an infiltration of polymorphonuclear leukocytes, mainly neutrophils, then a rapid influx of monocytes that develop into inflammatory macrophages that multiply rapidly. Cardinal signs of inflammation are dolor (pain), tumor (swelling), calor (heat) and rubor (redness) (Riciotti and Fitzgerald, 2011).

RA is characterised by a cytokine imbalance, with high concentrations of pro-inflammatory cytokines and low concentrations of anti-inflammatory cytokines. In RA, inflammatory cellular components penetrate the synovium which cause cartilage damage through the production and initiation of cytokines and other cells (Figure 1.2). The different cell types involved are responsible for the production of various cytokines including the interleukin (IL) family and tumour necrosis factor-alpha (TNF-α). Cytokines such as IL-23, IL-12, IL-15, and IL-18 are produced by dendritic cells (Gibofsky, 2012). T-cells are activated and T-helper lymphocytes are employed in the RA joint, of which Th17 cells (T-helper 17 cells) expresses cytokines such as IL-21, IL-17F, IL-22, IL-17A, and TNF-α. Cytokines such as IL-1β, IL-6, IL-21, and IL-23 are expressed by macrophages and dendritic cells leading to a high inflammatory state in the joint (Gibofsky, 2012). IL-23, IL-6, TNF-α, IL-1, IL-18, IL-12, and IL-15 have all been determined to be produced by macrophages which are linked to expressing MMPs that have a role in joint destruction (Gibofsky, 2012).

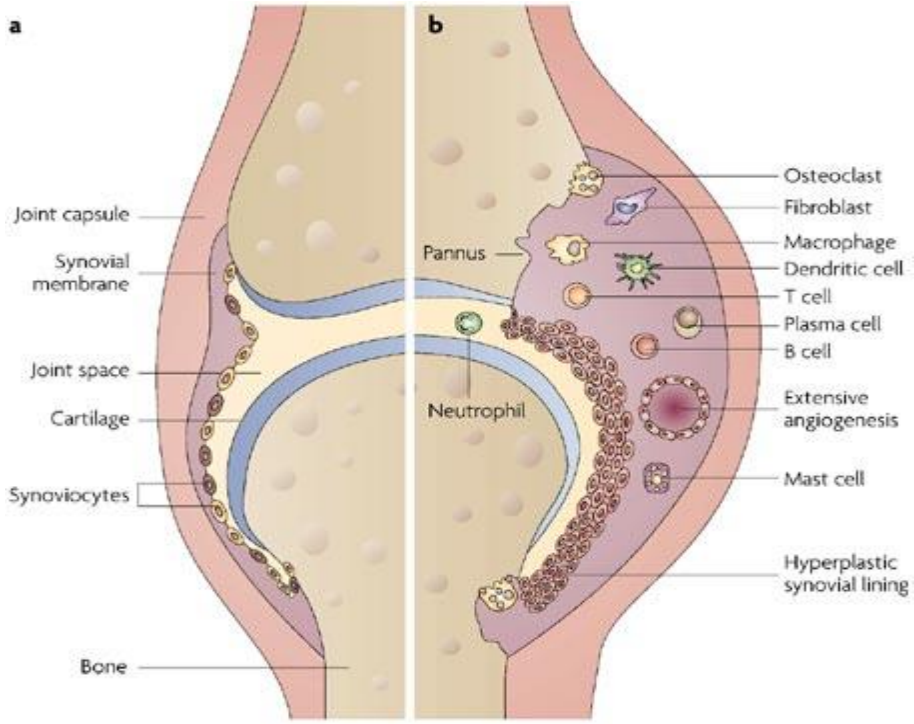


Figure 1.1: a) Normal joint; b) RA joint (Strand et al., 2007). a) Normal joint consists of bone enclosed with hyaline cartilage within a fibrous capsule; b) RA joint is characterised by the inflamed synovium spreading into the joint space eventually leading to joint destruction (Minnock, 2012). RA, rheumatoid arthritis.

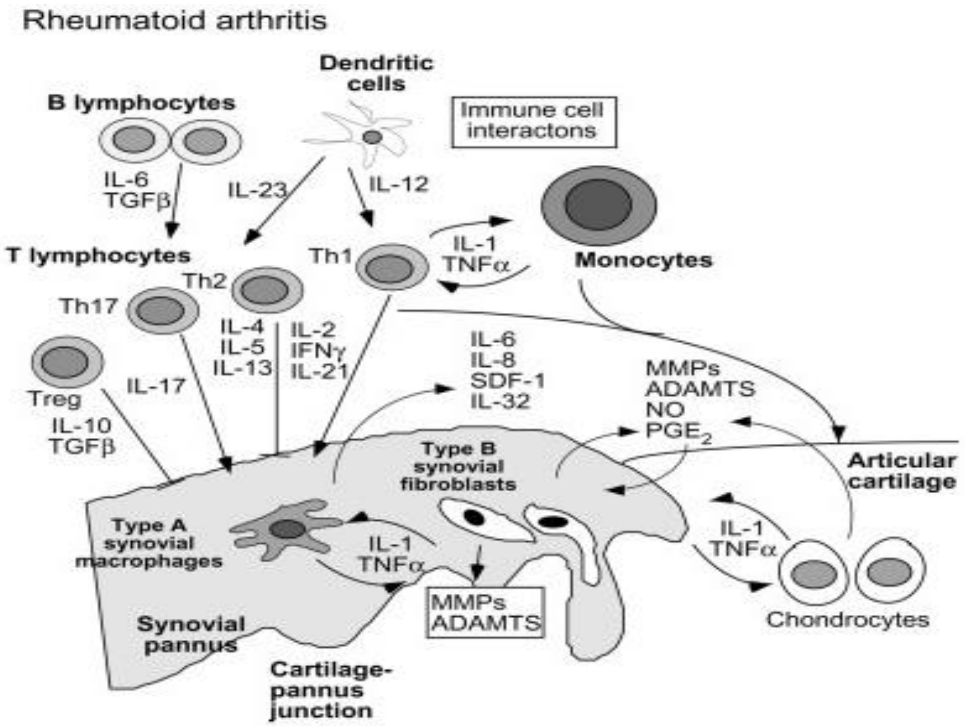


Figure 1.2: Overview of cytokine initiation and perpetuation in the RA joint (Goldring and Marcu, 2009a). Pro-inflammatory cytokines contribute greatly to the destruction of joint tissue by producing elevated concentrations of matrix metalloproteinases that degrade cartilage (Goldring and Marcu, 2009b). RA, rheumatoid arthritis; MMP, matrix metalloproteinase; IL, interleukin; TNF-α, tumour necrosis factor- alpha; ADAMTS, a disintegrin and metalloproteinase with thrombospondin-1 domains; TGF-β, transforming growth factor-beta.

1.2.3 Cytokine concentrations in rheumatoid arthritis

Previous research studies have determined the cytokines, TNF-α and IL-1 (IL-1α and IL-1β), play key roles in the cytokine cascade in RA. These two cytokines have been detected in blood, SF and joint tissue using techniques such as the ELISA assay (Burska *et al.*, 2014). Other cytokines associated with RA include IL-15, IL-18, IL-17, IL-7, IL-8 and IL-21. When the key pro-inflammatory cytokines (IL-6, IL-1α, IL-1β and TNF-α) are elevated in RA sufferers, there are also anti-inflammatory cytokines present (IL-4, IL-10 and IL-13) (Burska *et al.*, 2014). There are limitations to using cytokines as biomarkers for RA that must be considered. The interaction between cytokines is complex where a high concentration of a certain cytokine may cause the inhibition of another cytokine (Burska *et al.*, 2014). The cytokines, IL-1 (IL-1α, IL-1β and IL-1RA), IL-6 and TNF-α are not linked directly to a particular disease type, but rather to the cellular inflammatory process that takes place. This has made it difficult to find suitable cytokines to use as biomarkers for RA (Burska *et al.*, 2014).

Sivalingam *et al.* (2007) analysed multiple cytokines in the serum of RA patients using the ELISA assay, where the pro-inflammatory cytokines tested (IL-1β, IL-6, IL-8, IL-18 and TNF-α) were significantly increased in RA sera compared to healthy controls, while anti-inflammatory cytokines (IL-4 and IL-10) concentrations were low in the RA sera (Sivalingam *et al.*, 2007), thus reflecting the inflammatory state of RA. Abdullah *et al.* (2013) analysed IL-17 and IL-6 in the serum of RA patients using the ELISA assay. IL-17 concentrations were significantly elevated in RA patients as opposed to the healthy control group (median of 154.5 to 111.1pg.mL^{-1}, respectively) (Abdullah *et al.*, 2013). IL-6 was significantly elevated in RA patients compared to the healthy control group (median of 150 to 49pg.mL^{-1}, respectively) (Abdullah *et al.*, 2013). Lettesjo *et al.* (1998) analysed SF specimens for IL-1α, IL-1β, IL-6, IL-10, transforming growth factor-beta (TGF-β) and TNF-α in RA patients comparing them to a control group consisting of patients with polyarthritis, juvenile chronic arthritis, traumatic injury, and PSA. IL-1β, TGF-β, IL-6 and IL-10 were significantly higher in the RA patients (Lettesjo *et al.*, 1998).

1.3 Osteoarthritis

1.3.1 Overview of osteoarthritis

OA is the most common type of arthritis in the world, affecting twenty to thirty percent of the population (Clarkson *et al.*, 2016). OA is most prevalent in older people over fifty years old, with more female sufferers than male. Knee osteoarthritis (KOA) is the most common form of OA. The occurrence of KOA is two times greater than hip or hand OA (Cooper *et al.*, 2014). There is twice the number of females than males that suffer from hip, knee and hand OA (Cooper *et al.*, 2014). OA is usually diagnosed by general practitioners, who are mainly responsible for treating their condition (Clarkson *et al.*, 2016). To date, the aim in clinical management of this disease is controlling the symptoms encountered (Clarkson *et al.*, 2016). RA sufferers have elevated concentrations of erythrocyte sediment rate (ESR) and C-reactive protein (CRP), while in OA the concentrations are normally not elevated (Bondeson, 2015). OA is commonly non-inflammatory in nature, although it can have inflammatory elements (Bondeson, 2015). Research has suggested that the form of synovitis that occurs is operated and perpetuated by macrophages, which is seen in early OA (Bondeson, 2015).

1.3.2 Treatment of osteoarthritis

The synovitis experienced in OA fluctuates from patient to patient, with many suffering from an advanced inflammatory form of the disease (Bondeson, 2015). Arthrocentesis or steroid injectable drugs can significantly improve the quality of life of these patients (Bondeson, 2015). Drug treatments aim to relieve symptoms encountered in OA and target inflammatory components involved in the disease (Rainbow *et al.*, 2012). Drug treatment available for early OA include non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are popular but they have limitations such as: they do not halt the development of the disease and their effectiveness is limited and questionable with little differences between NSAIDs and placebo treatments reported (Rainbow *et al.*, 2012). Another treatment for OA is the use of disease modification OA drugs (DMOADs) that target pro-inflammatory mediators (Rainbow *et al.*, 2012).

Both MMPs and cytokines are targets for the development of DMOADs in clinical research (Karsdal *et al.*, 2016). MMPs have been linked to joint destruction in OA, especially MMP-13. These MMPs have become targets for disease modification in OA (Karsdal *et al.*, 2016). Examples of broad range MMP inhibitors where clinical studies were conducted include PG-116800 and BAY129566. However, these inhibitors were unsuccessful as effective treatments for OA (Karsdal *et al.*, 2016). MMP-13 targeted inhibitors are ongoing in clinical trials such as CP-544439, AZD-8955 and WAY-170523 (Karsdal *et al.*, 2016). Key pro-inflammatory cytokines (IL-1α, IL-1β, IL-6 and TNF-α) are becoming targets for DMOAD development and the clinical development of therapeutic agents (Karsdal *et al.*, 2016). Other types of DMOADs that have received interest in clinical studies include biphosphonates, calcitonin aggrecanase inhibitors, and estrogen (Karsdal *et al.*, 2016).

1.3.3 Pathophysiology of osteoarthritis

There have been three core phenotypes or subgroups identified in the pathophysiology of OA: (i) bone driven; (ii) inflammation driven; and (iii) cartilage and trauma driven (Karsdal *et al.*, 2016). OA is characterised by specific areas of damage to the joint surface, specifically the articular cartilage (Bondeson, 2015). Alterations in the KOA joint occurs (Figure 1.3) with formation of bone cysts (Man and Mologhianu, 2014). Histological assessment of the OA synovial membrane reveals hyperplasia of the lining layer, with an infiltration of cells, especially macrophages (Figure 1.4) (Bondeson, 2015). In healthy individuals, articular tissue consists of chondrocytes and extracellular matrix. The matrix is composed of water (H_2O), collagen, calcium and proteoglycans (Man and Mologhianu, 2014). The cellular signalling involved in cartilage destruction appears to be chondrocyte dependant (Goldring and Marcu, 2009b). The function of the chondrocytes is to produce mediators and proteolytic enzymes for dissolving matrix components (Man and Mologhianu, 2014). The mediators produced on pro-inflammatory pathways in OA are cytokines, chemokines, adipokines, damage-associated molecular patterns (DAMPs), ECM proteins, ECM degradation products, nitric oxide and toll-like receptors (TLR) ligands (Goldring and Otero, 2011).

Elements that affect chondrocyte activity include the matrix components themselves and cytokines present, mainly IL-1, TNF-α and IL-6 (Figure 1.4). OA results from the dysregulation between formation and breakdown of the mediators within the extracellular matrix, whereby the process is not in homeostasis (Man and Mologhianu, 2014). Proinflammatory cytokines, including IL-6, IL-1 and TNF-α, are produced in the collagen and proteoglycan degenerative process. These cytokines, in turn, play a key role in OA by attaching to chondrocytes thereby promoting the production of matrix metalloproteinases and a decrease in chondrocyte concentration due to a higher incidence of programmed cell death (apoptosis), ultimately leading to joint destruction (Man and Mologhianu, 2014).

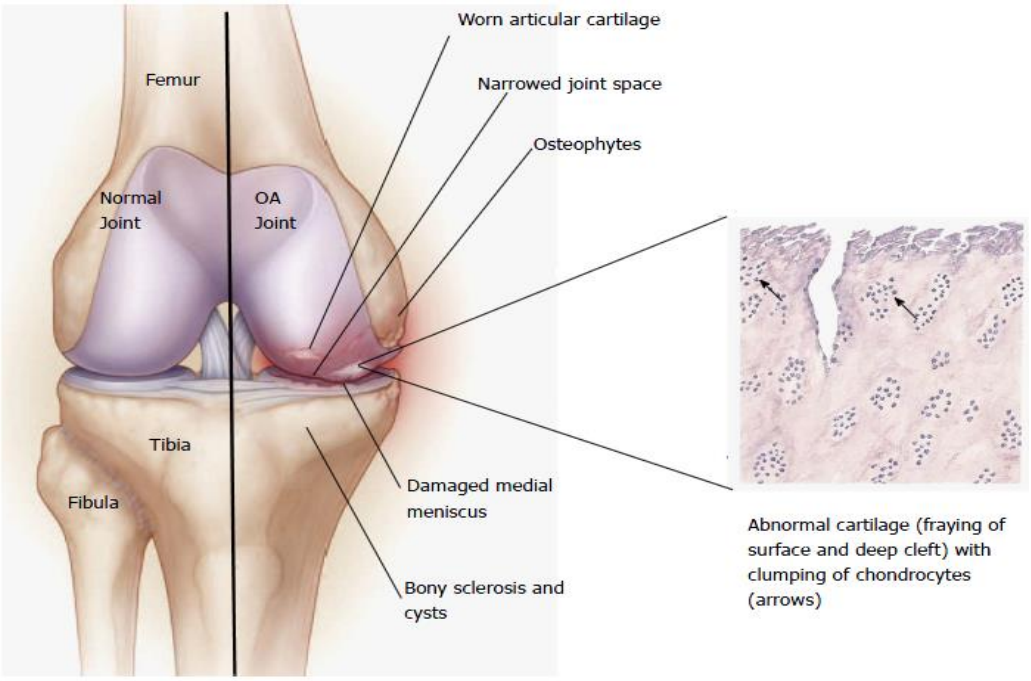


Figure 1.3: Overview of KOA in the joint (Uth et al., 2014). The diagram depicts a healthy and diseased OA joint. Subchondral bone alterations in OA include production of bone cysts, bone marrow lesions and sclerotic changes. Meniscus destruction occurs and new bone is formed at joint edges, due to presence of osteophytes (Man and Mologhianu, 2014). OA, osteoarthritis; KOA, knee osteoarthritis.

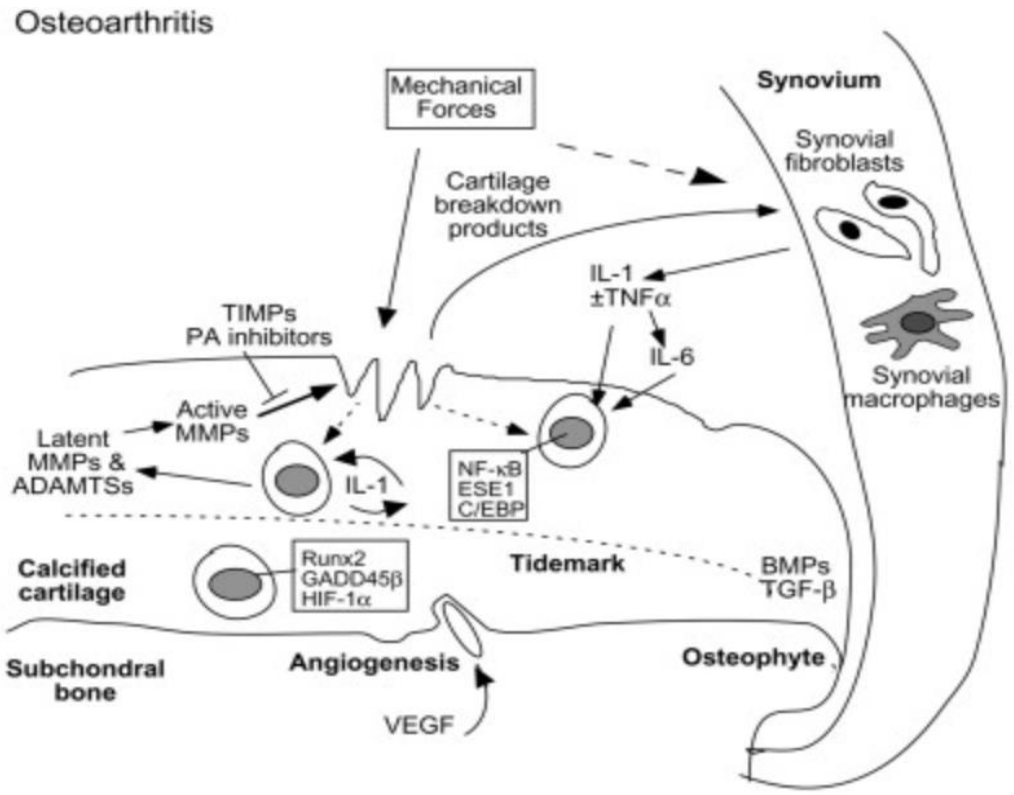


Figure 1.4: Overview of chondrocytes and key cytokines in the OA joint (Goldring and Marcu, 2009c). OA cartilage destruction occurs due to traumatic tissue insult, which is followed by inflammatory signalling with the production of key cytokines such as IL-1, IL-6 and TNF-α and MMPs by various cell types and chondrocytes in the joint (Goldring and Marcu, 2009b). MMP, matrix metalloproteinase; TGF-β, transforming growth factor-beta; IL, interleukin; TNF-α, tumour necrosis factor-alpha; VEGF; vascular endothelial growth factor; ADAMTS, a disintegrin and metalloproteinase with thrombospondin-1 domains; TIMPs, tissue inhibitors of metalloproteinases; PA, plasminogen activator.

1.3.4 Knee osteoarthritis diagnosis and surgery

Diagnosis of KOA is based on medical history, examination and analysis by radiography (Lewis and Carey Smith, 2012). Plain X-rays are used as a visual tool, in order to identify the classic signs of OA in the joint. These are the development of osteophytes, subchondral sclerosis, joint cavity narrowing and subchondral cysts (Lewis and Carey Smith, 2012). The condition is treated without surgery when a diagnosis is made. However, surgery is considered when all other therapeutic interventions have been exhausted. Many factors have to be explored before deciding on a particular surgery for a patient. Key factors are the prognosis of KOA and if the patient has an active lifestyle (Lewis and Carey Smith, 2012). Consultation with an orthopaedic surgeon determines when a patient is ready for surgery. Two common types of surgery include key-hole surgery and knee replacement surgery. Key-hole surgery (or arthroscopy) can be useful to visualise the internal articular knee joint. An arthroscope with a camera is inserted into small holes in the knee to examine the joint and other medical devices can be inserted for KOA therapeutic intervention. Joint replacement surgery (also known as arthroplasty) is employed in severe cases and in late-stages of KOA (Lewis and Carey Smith, 2012).

In the current study, posterior prosthesis (PS) or cruciate retaining (CR) prosthesis variants of Triathlon^{TM} (Figure 1.5) were used in total knee replacement (TKR) surgeries performed. The posterior cruciate ligament (PCL) restricts the posterior movement of the femur onto the tibia, which is important for knee stability. In patients undergoing TKR surgery, a decision has to be made to either retain the PCL by using a CR prosthesis or to surgically remove the PCL and use a PS prosthesis (Molt and Toksvig-Larsen, 2014). Molt and Toksvig-Larsen (2014) determined in their study that PS and CR variants of Triathlon^{TM} total knee have similar fixation capability over a two year period and predicted their performance would be similar over-time (Molt and Toksvig-Larsen, 2014). The techniques for TKR surgeries have been developed but fifteen to twenty percent of patients are unhappy with their outcome. Problems encountered by patients are knee stiffness and pain, loss of natural movements in the knee and loss of pre-surgery functions (Parcells and Tria, 2016a).

1.5a. Triathlon™ CR total knee prosthesis.

1.5b. Triathlon™ PS total knee prosthesis.

Figure 1.5a: Triathlon™ CR total knee prosthesis; 1.5b: Triathlon™ PS total knee prosthesis (Parcells and Tria, 2016b). Two commonly employed modern designs for total knee replacement surgery. The CR design was believed to retain the PCL which gave a more functional knee, while the PS was used in cases where the PCL could not be saved (Parcells and Tria, 2016a). CR, cruciate-retaining; PS, posterior-stabilised; PCL, posterior cruciate ligament.

1.3.5 Cytokines and matrix metalloproteinases in osteoarthritis

Biomarkers have been linked to pain classification of patients with OA so cytokine profiles may be useful to obtain information for this disease (Imamura *et al.*, 2015). Cytokines can be assessed to determine individuals at high-risk of developing KOA, where techniques such as radiography have failed (Mabey and Honsawek, 2015). OA is commonly graded radiographically based on the Kellgren and Lawrence (KL) grading system. This system has the following grades: grade 0, no radiological changes (no OA present); grade 1, unlikely narrowing of joint cavity and probable osteophytic lipping; grade 2, osteophytes are present with possible narrowing of joint cavity (OA is present but not severe); grade 3, several osteophytes present with definite joint cavity narrowing, some sclerosis and potentially bone deformity; grade 4, large osteophytes, obvious narrowing of joint cavity, extreme sclerosis with definite bone deformity (advanced OA) (Chen *et al.*, 2014). Cytokines associated with the advancement of KOA, particularly IL-6 and TNF-α have received considerable interest (Larsson *et al.*, 2015). Larsson *et al.* (2015) studied patients that appeared to have a different pathogenic process with increased joint reaction forces in development of their OA rather than an inflammatory mediated process (Larsson *et al.*, 2015). This illustrated OA can be classified as a pathogenic process or inflammatory process.

The study of cytokines has resulted in a greater understanding of the pathophysiology of OA. However, FDA accredited disease-modifying drugs based on cytokine manipulation for OA does not exist at present (Malemud, 2015). The inflammation involved with OA changes at different stages of the disease, with different cytokine profiles seen in early OA than later advanced stages (Mabey and Honsawek, 2015). Pro-inflammatory cytokines such as TNF-α and IFN-γ stimulate the production of IL-6, a major pro-inflammatory cytokine in OA. IL-6 ceases type II collagen manufacture in animal based studies (Mabey and Honsawek, 2015). IL-6 has been proposed as a biomarker for the early diagnosis of OA. A limiting factor to the success of IL-6 as a biomarker is that it is affected by circadian rhythms. This could lead to inaccurate diagnosis of OA due to excess concentrations of IL-6 present due to the circadian rhythms (Mabey and Honsawek, 2015). TNF-α may be useful as a therapeutic effectiveness marker and possibly a marker for disease affliction (Mabey and Honsawek, 2015).

The IL-17 class of interleukin are pro-inflammatory in nature and have been suggested as biomarkers for indicating pathogenesis of OA. This interleukin group contains six types, IL-17A to IL-17F. All forms of IL-17 are elevated in the SF and serum of OA patients (Wojdasiewicz *et al.*, 2014). IL-17 causes chondrocytes to stop manufacturing proteoglycans and stimulates MMP production. IL-17 initiates the production of IL-1β, TNF-α and IL-6 (Wojdasiewicz *et al.*, 2014). IL-10 is an anti-inflammatory cytokine that inhibits the manufacture of MMPs. IL-10 inhibits the effect of TNF-α in OA fibroblasts, thus illustrating the importance of this anti-inflammatory cytokine (Wojdasiewicz *et al.*, 2014). IL-13 is another anti-inflammatory cytokine that has been inhibits the production of IL-1β, TNF-α and MMP-3 (Wojdasiewicz *et al.*, 2014). IL-13 may be used as a therapeutic agent for OA as it inhibits pro-inflammatory cytokines while preserving chondrocytes (Wojdasiewicz *et al.*, 2014).

There are many factors that come into play in the destruction of the articular joint, where the presence of MMPs, in particular MMP-1, MMP-2, MMP-9, MMP-3 and MMP-13, have major roles (Malemud, 2015). Other enzymes that play a major role in OA are the disintegrin and metalloproteinases (ADAMS) and disintegrin and metalloproteinase with thrombospondin motif (ADAMTS). ADAMTS-4 and ADAMTS-5, for example, are important in the early stages of OA as they promote the destruction of proteoglycans and aggrecan (Malemud, 2015). MMPs and TIMPs are up-regulated in the SF of many OA sufferers (Orlowsky and Kraus, 2015). MMPs have been linked to OA pathogenosis. Zeng and Chen (2015) determined in their study that MMP-1, MMP-2 and MMP-9 concentrations were elevated in OA sufferers when compared to the healthy individuals. This study used meta-analysis on 10 published studies and suggested that MMP-1, MMP-2 and MMP-9 may be suitable markers for diagnosing and developing treatments for OA (Zeng and Chen, 2015). MMP-2 and MMP-9 concentrations in sera were elevated in OA patients, while MMP-1 and MMP-9 were elevated in SF of OA patients when compared to healthy individuals (Zeng and Chen, 2015).

1.3.6 Cytokine concentrations in knee osteoarthritis

Stannus *et al.* (2010) analysed serum for pro-inflammatory cytokines (IL-6 and TNF-α) from a study group of 172 participants (81 female, 91 Male), mean age of 63 years with an age range from 52 to 78 years, where 163 participants gave full cytokine profile data. The median concentration of cytokines determined using immulite immunometric assays was 2.9pg.mL^{-1} for IL-6 and 7.3pg.mL^{-1} for TNF-α (Stannus *et al.*, 2010). High IL-6 and TNF-α concentrations with increased joint space narrowing (JSN) were determined in older participants (Stannus *et al.*, 2010). The study presented an interesting statistic; sufferers with a low IL-6 concentration (found in the study to be ≤ 2pg.mL^{-1}) and low constant cartilage loss rate (0.6% per year) would never lose enough cartilage content to be classified as end-stage OA (i.e. sixty percent of cartilage is lost). However, with a high IL-6 concentration (≥ 4pg.mL^{-1}, as determined by the authors) and a 4.1% loss in cartilage per year, the process would only take fifteen years (Stannus *et al.*, 2010). This statistic indicated that an elevated serum IL-6 concentration ultimately leads to end-stage OA that requires surgery. This suggested that serum IL-6 and TNF-α play an important role in cartilage loss in the beginning of KOA (Stannus *et al.*, 2010).

Larsson *et al.* (2015) demonstrated that IL-6 and TNF-α in SF was linked to radiographic evidence of KOA in patients that have had menisectomy previously. There were 71 patients that donated SF samples at two different time points for cytokine analysis. The time between collections had a mean of 7.5 years with a range of 4 to 10 years and the time since their menisectomy had a mean of 18 years with a range of 15 to 22 years (Larsson *et al.*, 2015). Both IL-6 and TNF-α were analysed using a multiplex immunoassay and concentrations increased from the first assessment: TNF-α at first assessment contained a median of 3.13pg.mL^{-1}, on second assessment it contained 5.07pg.mL^{-1}; IL-6 contained a median of 1.86pg.mL^{-1} at first assessment, on second assessment it contained 5.10pg.mL^{-1} (Larsson *et al.*, 2015). The TNF-α concentration was associated with the advancement of OA based on this study and as a factor that precedes the condition (4 to 10 years); for every pg.mL^{-1} increase in the level of TNF-α, the risk increases for JSN and disability, with an increased risk of 1.7- and 1.5-fold, respectively (Larsson *et al.*, 2015). IL-6 and TNF-α concentrations in SF was linked with a higher risk of developing radiographic elements of KOA (Larsson *et al.*, 2015).

Imamura *et al.* (2015) recruited 101 participants (53 KOA patients and 48 healthy controls). The mean age ± standard deviation (mean ± SD) of KOA patients (all female) was 71.23 ± 7.62 years, while healthy group (2 male and 46 female) was 68.21 ± 7.17 years. Serum samples were analysed for IL-6, IL-8, TNF-α and IL-10 using a cytometric bead array by BDTM bioscience. IL-6 and IL-10 concentrations were significantly elevated in KOA patients, while IL-8 and IL-10 were similar between the groups (Table 1.1) (Imamura *et al.*, 2015). A large cytokine study was conducted using a Luminex multiplex bead array to analyse 21 cytokines, including IL-6, IL-10, IL-13, TNF-α and interferon-gamma (IFN-γ) (Tsuchida *et al.*, 2014). There were 27 KOA patients undergoing TKR surgery that donated SF to the study with a mean age of 70 years and range of 53 to 81 years. Healthy SF and cartilage was obtained from donors within 24 hours post mortem (n=20 with mean age of 40 years and range of 25 to 47 years) (Tsuchida *et al.*, 2014). Many of the cytokines analysed were higher in KOA patients than healthy controls, including IL-6, IL-8, IL-10, IL-13, TNF-α and IFN-γ (Table 1.1). IL-6 was significantly elevated in OA SF compared to the healthy controls (Table 1.1) (Tsuchida *et al.*, 2014).

Chen *et al.* (2014) analysed IL-17 in SF and serum of 100 KOA patients (21 male, 79 female) (Table 1.1), linking the cytokine with progression of the disease. The mean age was 61.98 years with a SD of 7.18 years and range of 40 to 75 years for the patient group. The healthy control group consisted of 50 individuals (10 male, 40 female) with a mean age of 58.58 years with a SD of 7.61 years and range of 40 to 75 years. IL-17 was only determined in KOA patients SF due to unavailability of SF in the healthy group (Chen *et al.*, 2014). Only patients with a KL grade of 2 or greater allowed in the study. The study reported a significant difference ($P<0.05$) between the IL-17 serum concentration in the KL grade 4 group (n=29) versus the grade 2 (n=34) and grade 3 (n=35) groups. The mean ± SD serum concentrations reported were as follows: grade 2 was 4.41 ± 0.75pg.mL^{-1}; grade 3 was 4.474 ± 0.816pg.mL^{-1}; grade 4 was 6.16 ± 1.12pg.mL^{-1}. Grade 3 and 4 had significantly higher IL-17 concentrations in SF than grade 2: grade 2 mean ± SD concentration was 5.57 ± 1.66pg.mL^{-1}, grade 3 was 6.73 ± 1.52pg.mL^{-1}, and grade 4 was 8.70 ± 2.15pg.mL^{-1}. The study reported a positive correlation between KOA severity (KL grade) and IL-17 concentration (Chen *et al.*, 2014) which suggested IL-17 may be useful as a biomarker for KOA.

Table 1.1: Cytokine concentrations in KOA studies that analysed serum and SF specimens (Imamura et al., 2015; Tsuchida et al., 2014; Chen et al., 2014). Healthy SF was obtained from donors (n=20) within 24 hours of post-mortem, defect SF was obtained from donors undergoing either microfracture or chondrocyte implantation (n=31) and OA patients (n=27). Differences in SF cytokine concentrations between healthy, cartilage defect and OA donors was presented (P<0.05) (Tsuchida et al., 2014). SF, synovial fluid; IL, interleukin; TNF-α, tumour necrosis factor-alpha; KOA, knee osteoarthritis; IFN-γ: interferon-gamma; pg.mL^{-1}, picograms per millilitre; P-value, statistical significance; SD, standard deviation; mean ± SD, mean ± standard deviation.

Cytokine	Group	mean ± SD (pg.mL^{-1})	*P*-value	Specimen	Study
TNF-α	KOA	2.20 ± 4.52	0.46	Serum	Imamura *et*
	Normal	1.40 ± 1.00		Serum	*al.*, 2015.
IL-10	KOA	1.58 ± 2.36	0.03	Serum	Imamura *et*
	Normal	0.89 ± 0.87		Serum	*al.*, 2015.
IL-6	KOA	4.38 ± 4.61	0.03	Serum	Imamura *et*
	Normal	2.55 ± 1.06		Serum	*al.*, 2015.
IL-8	KOA	10.56 ± 8.51	0.98	Serum	Imamura *et*
	Normal	9.72 ± 5.28		Serum	*al.*, 2015.
IL-17	KOA	4.17 ± 0.64	<0.05	Serum	Chen *et al.*,
	Normal	5.29 ± 1.47		Serum	2014.
IL-1α	KOA	15.00 ± 22.00	0.09	SF	Tsuchida *et*
	Normal	16.00 ± 10.00		SF	*al.*, 2014.
	Defect	14.00 ± 8.00		SF	
IL-1β	KOA	8.00 ± 16.00	0.03	SF	Tsuchida *et*
	Normal	1.00 ± 2.00		SF	*al.*, 2014.
	Defect	15.00 ± 18.00		SF	
IL-6	KOA	396.00 ± 508.00	<0.01	SF	Tsuchida *et*
	Normal	64.0 ± 120.00		SF	*al.*, 2014.
	Defect	261.00 ± 385.00		SF	
IL-8	KOA	52.00 ± 95.00	0.35	SF	Tsuchida *et*
	Normal	25.00 ± 29.00		SF	*al.*, 2014.
	Defect	27.00 ± 33.00		SF	
IL-10	KOA	9.00 ± 35.00	0.13	SF	Tsuchida *et*
	Normal	1.00 ± 6.00		SF	*al.*, 2014.
	Defect	0.00 ± 0.00		SF	
IL-13	KOA	18.00 ± 40.00	<0.01	SF	Tsuchida *et*
	Normal	1.00 ± 2.00		SF	*al.*, 2014.
	Defect	38.00 ± 41.00		SF	
TNF-α	KOA	4.00 ± 20.00	0.32	SF	Tsuchida *et*
	Normal	0.00 ± 0.00		SF	*al.*, 2014.
	Defect	2.00 ± 8.00		SF	
IFN-γ	KOA	51.00 ± 69.00	<0.01	SF	Tsuchida *et*
	Normal	47.00 ± 17.00		SF	*al.*, 2014.
	Defect	68.00 ± 38.00		SF	

1.4 Procedures and methods used in arthritis research

1.4.1 Efficient processing of blood specimens

The earliest question in an arthritis research project is what type of specimen (whole blood, serum or plasma) is required for cytokine analysis (Table 1.2) (Gillio-Meina *et al.*, 2013a). Blood processing refers to separating whole blood into plasma or serum using centrifugation. Whole blood may be required for some studies but usually serum or plasma is sufficient (Gillio-Meina *et al.*, 2013a). Once decided, a processing plan can then be developed. Various factors must be addressed when processing blood in order to obtain viable results. These include: a) selecting the correct collection tube for the study; b) suitability of the additives or coagulators applied to the walls of the tubes for the study; c) importance of eliminating or reducing the number of freeze-thaw cycles when storing the tubes; and d) importance of limiting time from collection to testing of the samples (Gillio-Meina *et al.*, 2013a).

Table 1.2: Processing procedure for blood (Gillio-Meina et al., 2013b). Collection, time to processing, time required for freezing, and freeze thaw procedures are essential in a study analysing blood (Gillio-Meina et al., 2013a).

Processing	**For Serum**	**For Plasma**
Collection (from whole blood).	Use the same collection tubes for each sample to reduce changes in analytes.	Use the same collection tubes for each sample to reduce changes in analytes.
Time to centrifugation.	30-60 minutes or longer if blood is treated with anticoagulants. Samples processed in <30 minutes can retain cellular components that may influence downstream analysis. Keep lag time before centrifugation constant to reduce sample variability.	Immediately. Keep lag time before centrifugation constant to reduce sample variability.
Time to freeze.	Immediately.	Immediately.
Storage.	-70^{o}C minimum to maximise storage duration without changes in sample quality.	-70^{o}C minimum to maximise storage duration without changes in sample quality.

1.4.2 Composition and collection of biological specimens

Human blood specimens are obtained by venepuncture. The liquid part of blood is plasma and it has the same colour and clarity as serum (clear to light yellow or deep yellow) (Bonewit-West, 2003). Blood is mainly composed of water (approximately ninety-two percent) with the other eight percent consisting of various dissolved proteins, clotting factors, hormones and glucose. High concentrations of dissolved proteins present in plasma include fibrinogen, prothrombin, serum albumin and globulins (Bonewit-West, 2003). Plasma without the clot factor fibrinogen is known as blood serum (Bonewit-West, 2003). The collection of SF is performed using a syringe in a process known as arthrocentesis or joint aspiration. There are a third less proteins present in SF than plasma as SF does not contain high-molecular weight components such as fibrinogen and β-2 and α-2 macroglobulin (Mundt and Shanahan, 2011). The highest amount of SF found in a healthy joint is 3.5mL, with a range of 0.1 to 3.5mL. The presence of the inflammatory response is indicated when SF concentrations exceed 3.5mL (Martinez-Castillo *et al.*, 2009).

Blood collection tubes are an essential part of a clinical study as they are coated with anti-coagulants and additives that have the ability to interfere with certain MMPs and cytokines (Gillio-Meina *et al.*, 2013a). Tubes used for serum collection usually contain clot coagulators that affect MMP and TIMP concentrations. The concentration of MMPs increase in serum tubes due to the presence of silicate salts or amorphous silica within clotting agents (Gillio-Meina *et al.*, 2013a). Tubes for plasma analysis contain anticoagulants such as ethylenediaminetetraacetic acid (EDTA) or sodium citrate. The concentrations of IL-10, IL-4, MMP-13, MMP-2 and MMP-1 increase due to EDTA. The EDTA tubes cause variations in cytokine profiles whereas sodium citrate treated tubes for plasma separation do not (Gillio-Meina *et al.*, 2013a). Mannello *et al.* (2003) recommended a buffered acidic citrate tube be used for plasma analysis, while tubes such as lithium heparin, dipotassium EDTA (K_2E), sodium fluoride potassium oxalate should be avoided as they affect MMP concentrations (Mannello *et al.*, 2003). The methods utilised for serum analysis are used for the analysis of SF (Mundt and Shanahan, 2011). Blood serum tube producers instruct leaving the blood for 30 to 60 minutes at room temperature (RT) to allow a clot to develop (Tuck *et al.*, 2009).

1.4.3 Cytokine assays used in arthritis research

Precision is "the closeness of agreement between independent test results under specified conditions" and there are 3 types: repeatability; intermediate precision; and reproducibility (Andreasson *et al.*, 2015). Selectivity is "the capability of a bioanalytical technique to measure and distinguish different analytes in the presence of components that may be expected to be present", where 2 terms are used: "selectivity" and "specificity". Selectivity can be scored on a scale while specificity cannot. Specificity is complete selectivity (Andreasson *et al.*, 2015). Several types of immunoassays are used in cytokine research (Table 1.3). The advantage of immunoassays is they have "good reproducibility" and "specificity", while their problem is "false positives" which have been attributed to heterophilic antibodies which often cause elevated cytokine concentrations in specimens (Burska *et al.*, 2014). The main drawback of ELISA is one cytokine can be analysed per kit, making multiple cytokine analysis very expensive. Multiplex systems are now available which can analyse several cytokines per kit (Burska *et al.*, 2014). The advantages of multiplex systems over ELISA assays are not as much sample is required (~20µL), conditions are similar, they are cheaper and faster, and broader information about the pathological process is obtained (Adamcova *et al.*, 2013).

Mass spectroscopy with peptide multiple reaction monitoring (MS-MRM) technology is an analytical technique that has provided another approach to analyse key proteins or cytokines in biological specimens (Ademowo *et al.*, 2013). The mass spectrometer is programmed to observe and track certain peptides which correlate to the concentration of specific proteins or cytokines in the specimen analysed (Ademowo *et al.*, 2013). The MRM technology allows for the determination of the quantity of protein or cytokine present by measuring the fragmented ions for the relevant peptide. The technique has a fantastic multiplexing capability (analyse over 25 peptides in one MRM protocol) with high accuracy and low-limits of detection. The technique is hoped to revolutionise biomarker analysis by boosting the amount of biomarkers used in clinical practice into the future (Ademowo *et al.*, 2013). Liquid chromatography tandem–mass spectrometry (LC-MS/MS) with MRM is a valuable technique for analysing key proteins or cytokines in complex specimens (Ademowo *et al.*, 2013).

Table 1.3: Overview of techniques used in cytokine research (Burska et al., 2014; Ademowo et al., 2013). Different immunoassays and a commonly used mass spectroscopy technique are summarised with key considerations for each. ELISA, enzyme linked immunosorbent assay; CV, coefficient of variance; LC-MS/MS, liquid chromatography tandem–mass spectrometry; qPCR, reverse transcriptase polymerase chain reaction; mRNA, messenger ribonucleic acid.

Assay	**Details**	**Considerations**
Immunassays.		
ELISA Assay.	-This assay involves the capture of the protein of interest by a primary antibody. -It is then detected by a secondary antibody followed by interpretation of results using an ELISA plate reader (Burska *et al.*, 2014).	-Not as sensitive as bioassays (<10pg.mL^{-1}). -Very good precision (CV: 5 to 10%). -Very easy and quick to perform. -Reagents are rather expensive. -Only analyse 1 cytokine per analysis.
Bioassay.	-These tests require the use of a tissue culture room. -The biological activity of the protein of interest is estimated by cellular proliferation using primary cell cultures (Burska *et al.*, 2014).	-It has good sensitivity (<1pg.mL^{-1}) but is non-specific. -Requires large amount of time and labour. -Poor precision (CV: 20 to 100%).
Molecular Forms.	- Include *qPCR*, microarrays and in-situ hybridisation. - Based on quantifying mRNA and require expensive equipment (Burska *et al.*, 2014).	-High precision, specificity and sensitivity. -Easy and quick to perform. -High overheads.
Mass-Spectroscopy.		
LC-MS/MS.	-Quantifies specific peptides from protein of interest (Ademowo *et al.*, 2013).	-Multiplexing capability. -No false positives whereas immunoassays have high number of false positives.

1.4.4 Zymography techniques used in arthritis research

There are several modifications of substrate zymography used for research purposes (Table 1.4) (Snoek-van Beurden and Von den Hoff, 2005). Substrate zymography is used for MMP identification based on their preferential substrate degradation and using molecular weight markers (Snoek-van Beurden and Von den Hoff, 2005). It is possible to determine if the MMP is in the active or latent form by using substrate zymography (Snoek-van Beurden and Von den Hoff, 2005). The bands of TIMPs are detected using reverse zymography (a form of substrate zymography) by using their MMP inhibition properties (Snoek-van Beurden and Von den Hoff, 2005). Gelatin zymography is the basis for all forms of substrate zymography (Snoek-van Beurden and Von den Hoff, 2005). The principles of zymography are: a. gelatin is retained in the gel during electrophoresis; MMP is inhibited by sodium dodecyl sulfate (SDS) using electrophoresis; and SDS causes MMP-TIMP complex to segregate during electrophoresis. The detection of individual MMPs and TIMPs is achieved using substrate zymography (Snoek-van Beurden and Von den Hoff, 2005).

Table 1.4: Zymographic techniques for the analysis of MMPs (Snoek-van Beurden and Von den Hoff, 2005). Several modifications of zymography have been developed to analyse different type of MMPs which have different sensitivities. MMP, metalloproteinase; TIMP, tissue inhibitor of metalloproteinase (Snoek-van Beurden and Von den Hoff, 2005).

Assay Type	**Principle**	**MMPs Detected**	**Sensitivity**
Gelatin Zymography.	Gelatin is the preferential substrate for MMP-2 and -9.	MMP-2 & MMP-9 (commonly detected). MMP-1, MMP-8, MMP-13 can be detected (Not preferential substrate-weak signal).	High. Up to 10pg of MMP-2 can be detected.
Casein Zymography.	Casein is a suitable substrate for the stromelysins.	MMP-1, MMP-7, MMP-12, MMP-11 and MMP-13 are detected. MMP-9 can be detected when it has high presence.	Lower than gelatin zymography.
Collagen Zymography.	Collagen is added to the zymogram which can be used as SDS disrupts the fibers of collagen allowing separation of proteins.	MMP-1 and MMP-13 are usually analysed using collagen. MMP-2 and MMP-9 are detectable.	10pg of pro-MMP-1 and 0.1pg of active MMP-1 can be detected.
Heparin-Enhanced Substrate Zymography.	MMP-7 & collagenases difficult to detect using casein or gel zymography. Heparin extraction of MMPs from samples cause higher MMP concentrations.	Suitable for MMP-7.	Intermediate & active MMP-7 detection increase ~5 fold in heparin. Detection of pro-MMP-7 increase by ~20 fold.
Reverse Zymography.	MMP (normally MMP-2) is added to the gel along with the gelatin. TIMPs inhibit MMP-2 & gelatin is not digested resulting in blue TIMP bands.	Suitable for 4 human TIMPs which are Timp-1, TIMP-2, TIMP-3 and TIMP-4.	Sensitivity is greatly enhanced from optimising the gel and by adjusting added MMP concentration.

1.5 Aims and objectives

The main aim of this research project was to investigate blood and aspirated SF specimens for pro- and anti-inflammatory cytokines and MMPs in KOA patients to determine if the disease was characterised as inflammatory or non-inflammatory in nature. The relationship or magnitude of difference in cytokine concentration between matched serum and SF samples from individual KOA patients was investigated.

This study set out to meet the following objectives:

- Investigate the profile concentrations of pro- and anti-inflammatory cytokines and MMPs in matched blood and SF specimens from patients with severe KOA undergoing TKR surgery.
- Utilisation of two molecular based techniques: gelatin zymography (for MMP-2 and MMP-9 concentration comparison in plasma samples); and the ELISA assay for cytokine quantification in serum and SF samples.
- Determine if there was a significant difference between the patient and healthy, asymptomatic group, for serum concentrations of pro-inflammatory (IL-6, TNF-α, IL-17, IFN-γ) and anti-inflammatory cytokines (IL-13 and IL-10) using SPSS statistical analysis (IBM SPSS Statistics Version 22).
- Determine if the cytokine profile (pro- and anti-inflammatory) in KOA patients, allowed for diagnostic assistance in the healthy, asymptomatic group.

Chapter 2: Materials and Methods

2.1 Informed consent and ethical approval

Ethical approval for the project was obtained from the ethics committee at the Institute of Technology Carlow and from AutEven Hospital in Kilkenny. Informed consent (Appendix I) was obtained from patients undergoing TKR surgery (arthroplasty) in AutEven Hospital and from healthy volunteers in the asymptomatic group from the Institute of Technology Carlow for participation in the current study. Detailed medical histories were not presented for patients and healthy volunteers in the study. However, the healthy volunteer group completed an assessment form (Appendix II) prior to participation in the study which indicated if they suffered from any form of arthritis or inflammatory disorder.

The study demographic included patients with severe KOA undergoing TKR surgery (Table 2.1) and healthy volunteers that have not been diagnosed with any form of arthritis (Table 2.2). The mean age ± SD of the patient group (n=30) was 68.77 ± 8.10 years, while the healthy volunteer group (n=23) was 27.74 ± 5.05 years. Selected healthy volunteers provided blood samples at two time points to assess the difference in certain cytokines over time. The selection criteria was based on availability of blood samples at the two time points. There was twenty months between the first (November 2014) and second blood collection (July 2015) for the healthy volunteer group. The presence of “a” after subject number indicated the blood sample was from the first collection, while “b” indicated that the blood sample was from the second collection. Each participant in the study was randomly assigned a unique number for identification purposes.

Table 2.1: Patient study demographic with surgery details. All patients had severe KOA to either the left or right knee. Patients donated matched blood and synovial fluid samples on the day of surgery. Triathlon™ PS or CR prosthesis were used in TKR surgeries. The code refers to the study identification number for each patient. CS, cruciate-substituting; TKR, total knee replacement; PS, posterior-stabilising; M, male; F, female; P, patient.

Code	Sex	Age (Years)	Surgery performed
P1	M	57	Right TKR: Triathlon™ CS TKR & patella.
P2	F	69	Complex primary left TKR: Triathlon™ CS TKR & patella.
P3	M	85	Left TKR: Triathlon™ CS TKR & patella.
P4	F	71	Right TKR: Triathlon™ CS TKR & patella.
P5	M	71	Left TKR: Triathlon™ CS TKR & patella.
P6	M	61	Right TKR: Triathlon™ CS TKR & patella.
P7	F	71	Left TKR: Triathlon™ CS TKR & patella.
P8	F	76	Left TKR: Triathlon™ CS TKR & patella.
P9	M	71	Right TKR: Triathlon™ CS TKR & patella.
P10	M	67	Right TKR: Triathlon™ CS TKR & patella.
P11	F	65	Left TKR: Triathlon™ CS TKR & patella.
P12	F	75	Left TKR: Triathlon™ CS TKR & patella.
P13	F	69	Left TKR: Triathlon™ CS TKR & patella.
P14	F	60	Left TKR: Triathlon™ CS TKR & patella.
P15	F	78	Left TKR: Triathlon™ CS TKR & patella.
P16	F	65	Left TKR: Triathlon™ CS TKR & patella.
P17	F	60	Complex primary right TKR: Triathlon™ PS TKR & patella.
P18	F	49	Right TKR: Triathlon™ PS TKR & patella.
P19	M	58	Left TKR: Triathlon™ PS TKR & patella.
P20	F	56	Complex primary left TKR: Triathlon™ CS TKR & patella.
P21	F	72	Right TKR: Triathlon™ CS TKR & patella.
P23	F	73	Right TKR: Triathlon™ CS TKR & patella.
P25	F	72	Right TKR: Triathlon™ CS TKR & patella.
P26	M	73	Complex primary right TKR: Triathlon™ PS TKR & patella.
P30	F	69	Left TKR: Triathlon™ PS TKR & patella.
P32	M	79	Right TKR: Triathlon™ PS TKR & patella.
P36	F	66	Left TKR: Triathlon™ PS TKR & patella.
P37	M	67	Right TKR: Triathlon™ PS TKR & patella.
P39	F	81	Left TKR: Triathlon™ CS TKR & patella.
P40	F	77	Left TKR: Triathlon™ CS TKR & patella.

Table 2.2: Healthy volunteer study demographic and their health status at the time of the study. All volunteers were healthy at the time of the study based on completion of the voluntary assessment forms. No volunteer declared that they suffered from any form of arthritis. The volunteer with the code, V2, suffered from juvenile arthritis but this never developed into adulthood. The code refers to the study identification number for each volunteer in the study. M, male; F, female; V, volunteer.

Code	**Sex**	**Age (Years)**	**Health status**
V1	F	32	Healthy at time of study.
V2	M	32	Healthy at time of study. Suffered from juvenile arthritis in childhood.
V4	M	36	Healthy at time of study.
V5	F	29	Healthy at time of study.
V6	F	24	Healthy at time of study.
V8	F	24	Healthy at time of study.
V9	M	25	Healthy at time of study.
V10	F	25	Healthy at time of study.
V11	M	30	Mild psoriasis sufferer. Otherwise, healthy at time of study.
V12	M	26	Healthy at time of study.
V13	F	25	Healthy at time of study.
V14	F	28	Healthy at time of study.
V15	F	27	Healthy at time of study.
V16	F	25	Healthy at time of study.
V17	F	22	Healthy at time of study
V18	F	26	Healthy at time of study.
V19	M	25	Mild psoriasis sufferer. Otherwise, healthy at time of study.
V20	M	25	Healthy at time of study.
V21	M	23	Healthy at time of study.
V23	F	34	Mild psoriasis sufferer. Otherwise, healthy at time of study.
V24	F	44	Healthy at time of study.
V25	F	26	Healthy at time of study.
V26	M	25	Healthy at time of study.

2.2 Sample processing and preparation

2.2.1 Blood processing for gelatin zymography and ELISA assays

Blood specimens were collected in Greiner VACUETTE® 3mL 9NC sandwich tubes with 3.2% sodium citrate (plasma tubes) and Greiner VACUETTE® 4mL Z serum sep clot activator gold tubes (serum tubes). Blood collection tubes were treated according to the manufacturer's specifications. The serum tubes were left to clot for at least 30 minutes after blood collection prior to centrifugation. The serum tubes were centrifuged at 1800rpm (revolutions per minute) for 10 minutes within 1 hour of blood collection, whereas the plasma tubes were centrifuged at 2800rpm for 15 minutes within 1 hour of blood collection using the VWR CompactStar CS4 centrifuge. The tubes had to be centrifuged within 2 hours of collection. Aliquots of 200µL of serum and plasma were placed into sterile eppendorfs and placed on ice until storing them at -80^{o}C until analysis (De Jager *et al.*, 2009; Hu and Beeton, 2010). Samples were thawed at ambient room temperature (RT) for 15 to 30 minutes prior to analysis. Serum tubes were used for ELISA analysis, while plasma samples were used both in gelatin zymography and ELISA assays.

2.2.2 Synovial fluid processing for the ELISA assays

Jayadev *et al*. (2012) described a procedure for SF ELISA analysis that was adapted in this study. SF was collected in Greiner VACUETTE® 4mL Z no additive white/black tubes and 200µL aliquots were placed into sterile eppendorfs. The eppendorfs with SF were spun at 6139rpm for 25 minutes at 4°C using the Hanil BiMed Inc. Model Smart R17 Centrifuge and the eppendorfs were stored at -80^{o}C until analysis (Jayadev *et al.*, 2012). Before the ELISA analysis, SF was thawed at RT on ice for 15 to 30 minutes and then centrifuged at 11,208rpm for 10 minutes. Hyaluronidase (type IV-S, 890U.mg^{-1}, Sigma) was used to treat SF prior to analysis to reduce viscosity (Jayadev *et al.*, 2012). This involved using a 1:1 dilution of the SF supernatant with 4mg.mL^{-1} hyaluronidase which was prepared with the sample diluents or assay diluents (Jayadev *et al.*, 2012) that was included in the particular cytokine ELISA kit being tested. This solution was mixed using the Lennox vortex mixer VM-10 model for 5 seconds, followed by shaking at RT for 1 hour at 400rpm (Jayadev *et al.*, 2012).

2.3 Gelatin Zymography

2.3.1 Reagent and solution preparation

The reagents and solutions for gelatin zymography were modified from those described by Frankowski *et al.* (2012) and Toth and Fridman (2001). The reagents and solutions used were made up as follows:

a. Tris solutions

- *1.5 mol.L^{-1} Tris-Cl, pH 8.8:* This solution was prepared by dissolving 18.15g trisaminomethane (Tris) in sterile deionised water (diH_2O). The pH was adjusted to pH 8.8 with 8.0 mol.L^{-1} hydrochloric acid (HCl) and made up to a final volume of 100mL. This solution was autoclaved for 15 minutes at 120^{o}C and stored at RT.
- *0.5 mol.L^{-1} Tris-Cl, pH 6.8:* This solution was prepared by dissolving 6.05g Tris in diH_2O. The pH of the solution was adjusted to pH 6.8 with 8.0 mol.L^{-1} HCl and made up to a final volume of 100mL. This solution was autoclaved for 15 minutes at 120^{o}C and stored at RT.

b. Polyacrylamide solution

30% polyacrylamide (1:29) was prepared by dissolving 29.2g acrylamide and 0.8g NN'-methylene-bis-acrylamide in 100mL diH_2O. This solution was filtered (0.45µm) and stored away from light at 4^{o}C for up to 1 month.

c. Ammonium persulfate (APS) solution

10% APS: 0.1g APS was made up to 1mL with diH_2O. This served as a catalyst for gel polymerisation. This solution was stored at 4^{o}C for up to 2 weeks.

d. Inhibition control incubation buffers

- *EDTA inhibition solution:* 8.766g sodium chloride (NaCl), 6.057g Tris, 1.47g calcium chloride dihydrate ($CaCl_2$), 0.5g sodium azide (NaN_3) and 5.845g EDTA was dissolved in 800mL diH_2O and further diluted with diH_2O to the 1000mL mark of a volumetric flask.
- *Incubation buffer without $CaCl_2$:* 8.766g NaCl, 6.057g Tris and 0.5g NaN_3 was dissolved in 800mL diH_2O and further diluted with diH_2O to the 1000mL mark of a volumetric flask. The pH was adjusted to 7.8 to 8.0 with 8.0 mol.L^{-1} HCl and was stored at 4^{o}C for 6 months.

e. Gelatin solution

Gelatin, from bovine skin, type B: 100mg gelatin, 4.5mL diH_2O, 0.5mL 10% SDS was mixed together in a plastic container. This was made fresh for each experiment.

f. Running buffer

5X running buffer: 15.1g Tris, 94g glycine and 5g SDS was dissolved in 800mL diH_2O and further diluted with diH_2O to the 1000mL mark of a volumetric flask. The pH was adjusted to 8.3 with 8.0 mol.L^{-1} HCl and the solution was stored at RT. To make 1X running buffer, 200mL of 5X buffer was further diluted with 800mL diH_2O to a final volume of 1000mL.

g. Gel washing buffer

10X gel washing Buffer: 25mL of Triton X-100 was diluted with 975mL diH_2O. The pH was adjusted to 7.8 to 8.0 with 8.0 mol.L^{-1} HCl and stored at RT. To make 1X washing buffer, 100mL of the 10X running buffer was dissolved in 1000mL diH_2O.

h. Gel development buffer (incubation buffer)

Incubation buffer (0.05 mol.L^{-1} Tris HCl, 0.15 mol.L^{-1} NaCl, 0.01 mol.L^{-1} $CaCl_2$, pH 8.0): 8.766g NaCl, 6.057g Tris, 1.47g $CaCl_2$ and 0.5g NaN_3 was dissolved in 800mL diH_2O and further diluted with diH_2O to the 1000mL mark of a volumetric flask. The pH was adjusted to 7.8 to 8.0 with 8.0 mol.L^{-1} HCl and the solution was stored at 4^oC for 6 months.

i. Staining solution

Coomassie blue R-250 stain: 0.125g coomassie R-250, 1mL acetic acid glacial, 45mL methanol and 54mL diH_2O were mixed together in a 100mL volumetric flask. This solution was filtered using a 0.45µm filter and stored at RT.

j. De-staining solution

Methanol: acetic acid: H_2O (50:10:40): 500mL methanol, 10mL acetic acid and 40mL diH_2O were mixed together in a 1000mL volumetric flask. This solution was filtered (0.45µm) and stored at RT.

k. <u>Sample buffer</u>

2X non-reducing sample buffer: 1mL 0.5mol/L Tris (pH 6.8), 0.8ml glycerol, 3.2mL 10% SDS and 0.2mL 0.2% bromophenol blue were mixed together using the Lennox vortex mixer VM-10 model. Aliquots were stored at -20^{o}C until analysis.

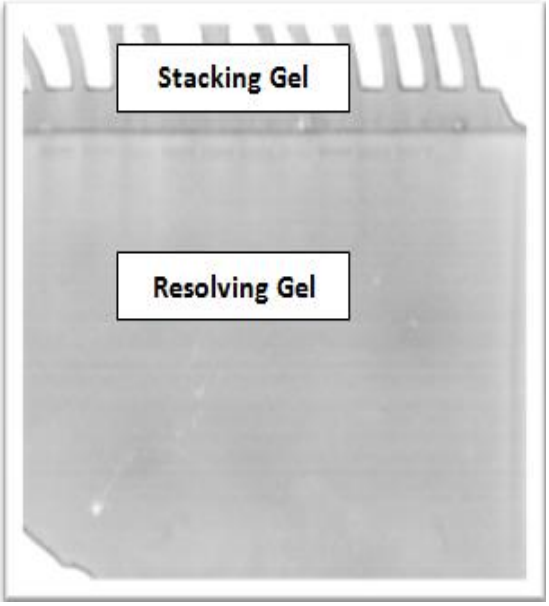
l. <u>Sodium dodecyl sulfate solution</u>

10% SDS: 10g SDS was dissolved in diH_2O. This was made up to 100mL with diH_2O. This was stable for 1 year at RT.

2.3.2 Gelatin zymogram preparation

The resolving and separating gels (Figure 2.1) were prepared using the recipe by *Kupai* (2011) outlined in Table 2.2. The gelatin zymogram was made fresh for each experiment using a modified version of the preparation assay as described by Frankowski *et al.* (2012). The preparation procedure was as follows:

- 15mL of 8% resolving gel (sufficient for 2 gels) was prepared. Sterile diH_2O, 1.5 mol.L^{-1} Tris, 10% SDS, 30% polyacrylamide and gelatin solution were mixed together in a sterile container (Starstedt 50mL SC Tube). This mixture was left for 3 minutes before APS and N,N,N',N'-Tetramethylethylenediamine (TEMED) were added, followed by mixing again using the Lennox vortex mixer VM-10 model.
- The resolving gel solution was poured into the gel casting chamber. A layer of diH_2O was placed over the resolving gel to prevent bubble formation and to keep the top of the resolving gel straight for adding the stacking solution. The gel was left to polymerise for 1 hour.
- 6mL of the 5% stacking gel (2 to 3 gels) was prepared: diH_2O, 0.5 mol.L^{-1} Tris, 30% polyacrylamide were mixed using the Lennox vortex mixer VM-10 model in a sterile container (Starstedt 50mL SC Tube). APS and TEMED were added lastly followed by mixing. The layer of diH_2O was removed from the resolving gel. The stacking gel was placed over the resolving gel with a sterile plastic comb inserted and allowed to stand for 15 to 20 minutes before removing the comb.



2.1a. ATTO 1D chamber with mini gel. 2.1b. Gelatin zymogram.

Figure 2.1a: the ATTO 1D chamber with gel electrophoresis mini gel (8x9cm); 2.1b: Gelatin zymogram (stacking and resolving gel). Setup of ATTO SDS PAGE unit with the gelatin zymogram. The ATTO 1D chamber with mini gel system used was the AE6450 model. This model allowed for 2 gels can be run in a single run.

Table 2.3: Stacking and separation gel recipes (Kupai, 2011). Gelatin zymogram for detection of MMP-2 and -9 was made using an 8% resolving gel with a 5% stacking gel (Kupai, 2011). SDS, sodium dodecyl sulfate; APS, ammonium persulfate solution; TEMED, N,N,N',N'-Tetramethylethylenediamine; diH_2O, sterile deionised water; MMP, matrix metalloproteinase; HCl, hydrochloric acid.

Resolving Gel 8% *Solution*	*Amount*	***Stacking gel 5%*** *Solution*	*Amount*
30% polyacrylamide	4mL	30% polyacrylamide	0.65mL
1.5M Tris HCl, pH 8.8	3.75mL	0.5M Tris HCl, pH 6.8	1.25mL
diH_2O	5.75mL	diH_2O	3.05mL
Gelatin (20mg.mL^{-1})	1.5mL	10% SDS	50µL
10% APS (100mg.mL^{-1})	50µL	10% APS	25µL
TEMED	10µL	TEMED	7µL

2.3.3 Gelatin zymography laboratory protocol

The laboratory protocol, adapted from methods described by Frankowski *et al.*, (2012), Toth and Fridman (2001); and Hu and Beeton (2010) was conducted as follows:

1. The prepared gels (as described in Section 2.3.2) were added into the electrophoresis chambers (2 gels per unit) and 1X running buffer (as described in Section 2.3.1) was added to ATTO 1D chamber (AE6450 model).
2. All samples, standards and protein ladders were loaded using StarLab 0.1-10µL round gel loading pipette tips (product code: I1010-3000). Plasma from patients and healthy volunteers in the study were used for gelatin zymography: 1µL plasma was added to 10µL 2X non-reducing sample buffer and 10µL of this mixture was added to the wells of the stacking gel in duplicate for each respective patient or volunteer tested. The BioChemica protein marker VI (10 to 245kDa) pre-stained protein ladder was added to the first and last well of the stacking gel (7µL of the ladder was sufficient) to aid with band size identification.
3. Bio-Techne recombinant human MMP-2 western blot standard and Bio-Techne MMP-9 western blot standard standards were added at a concentration of 10µL to the second and third well of the stacking gel to aid with MMP band identification.
4. The gels were run at 125V for 90 minutes or until the dye front with bromophenol blue tracking dye had travelled to the bottom of the resolving gel using the BioRad PowerPac™ basic power supply 10 to 300V.
5. The gels were removed from the glass plates, placed into plastic containers and washed with the gel wash solution (Triton X-100 solution). Each gel received 3 washes with gentle agitation for 15 minutes each time.
6. Gels were incubated for 42 hours at 37^{o}C.
7. The gel development buffer was removed, gels rinsed with diH_2O until foaming ceased and 100mL of the gel staining solution was placed in the plastic containers for 1 to 2 hours.
8. Gels were de-stained using the methanol based de-staining solution for 2 to 3 hours until clear bands could be observed.

9. Gels were placed in clear plastic acetate sheets and scanned on a standard office scanner with resolution of 300DPI. Band intensity measurements for gelatin zymograms were determined using NIH ImageJ Analysis Software for total MMP-9 comparison. Visually MMP-2 bands were observed on gelatin zymograms.

2.4 Enzyme linked immunosorbent assays

2.4.1 R&D systems human IL-6 ELISA kit

All reagents and standards were prepared as described in the technical sheet provided (Appendix III). The laboratory procedure for the R&D systems precoated IL-6 ELISA kit (catalogue #: D6050) was as follows:

1. The IL-6 standard was reconstituted with 5mL of calibrator diluent RD6F to make stock solution of 300pg.mL^{-1}. This solution was allowed to sit for 15 minutes with gentle agitation prior to making dilutions. A volume of 333µL stock was transferred to a tube in which 667µL of calibrator diluent RD6F was added to make the 100pg.mL^{-1} standard. 500µL of this solution was added to the next tube and 500µL of the calibrator diluent RD6F was added to make the 50pg.mL^{-1}standard. This was repeated for the 4 lower standards (25pg.mL^{-1}, 12.5pg.mL^{-1}, 6.25pg.mL^{-1} and 3.13pg.mL^{-1} standards). The calibrator diluent RD6F served as the zero standards (group blank).
2. The precoated ELISA plate was removed from the foil packaging and 100µL of assay diluent RD1W was added to each well.
3. A volume of 100µL standard and sample (plasma and serum from healthy individuals and KOA patients in the study) was added to each well in triplicate and the plate was covered with the adhesive strip provided. The plate was incubated at RT for 2 hours with gentle agitation.
4. The wells were aspirated and washed using the wash buffer solution 4 times. A volume of 400µL wash buffer was added to each well and removed by inversion with gentle tapping. This was followed by blotting the plate against paper towels to remove excess solution.

5. A volume of 200µL human IL-6 conjugate was added to each well and an adhesive strip was placed over the plate. The plate was incubated for 2 hours at RT.
6. The aspiration and washes performed in step 4 were repeated.
7. A volume of 200µL substrate solution was added to each well. This was incubated for 20 minutes at RT in a dark area.
8. A volume of 50µL stop solution was added to each well. The optical density of each well was determined at 450 and 570nm within 30 minutes using the VersaMax microplate reader from Molecular Devices with SoftMax Pro 6.2.2. Data generated was analysed using the 5-parameter curve fit setting. The resultant constants for the cubic equation were entered into a spreadsheet to assess the concentration of IL-6 of the unknown samples on each plate.

2.4.2 Affymetrix eBioscience Human IL-6 ELISA kit

Concentrations of IL-6 from serum and SF samples, respectively, were determined using a precoated ELISA kit (Affymetrix eBioscience human IL-6 platinum precoated ELISA kit, catalogue #: BMS213/2CE). All the reagents and working standards were prepared according to the procedures described in the technical sheet provided in the kit (Appendix IV). The laboratory procedure for the IL-6 ELISA was as follows:

1. The microwell strips were washed twice with approximately 400µL wash buffer per well with thorough aspiration between washes. The wash buffer was allowed to sit in the wells for 10 to 15 seconds before aspiration. This was removed by inversion with gentle tapping. This was followed by blotting the plate against paper towels to remove excess solution.
2. The standard dilution of the IL-6 standard was prepared on the microwell plate. A volume of 100µL of 1X assay buffer was added in duplicate to all standard wells. A volume of 100µL prepared standard (200pg.mL^{-1}) was added to wells A1 and A2. The contents were mixed with repeated aspiration and ejection, and 100µL was transferred to wells B1 and B2, respectively. This was repeated 5 times, creating 2 rows of human IL-6 standard dilutions, ranging from 100 to 1.56pg.mL^{-1}. A volume of 100µL

of the last dilution in the series (G1, G2) was discarded from the last microwells. A volume of 100µL 1X assay buffer was added in duplicate to H1 and H2 which served as the plate blank.

3. For the serum samples procedure, 50µL of assay buffer (1X) was added to all the sample wells. A volume of 50µL of each sample was then added in duplicate to the sample wells. For the SF samples procedure, 100µL of the hyaluronidase treated samples was added in duplicate. There were 2 dilutions tested for SF (1:2 and 1:8 dilutions). A high and low control was added in duplicate. The high control range was 100 to 200pg.mL^{-1} and the low control range was 5 to 15pg.mL^{-1}.
4. The biotin conjugate was prepared by adding 60µL of this solution to 5.94mL of 1X assay buffer. A volume of 50µL biotin conjugate was added to all wells. The plate was covered with adhesive film and incubated at RT for 2 hours on the Labnet Orbit 300 laboratory shaker set at 400rpm.
5. The streptavidin-horseradish peroxidase (streptavidin-HRP) solution was prepared by adding 60µL of streptavidin-HRP to 11.94mL of 1X assay buffer.
6. The adhesive film was removed from the plate and the wells were emptied. The microwell strips were washed according to step 1 in the procedure.
7. A volume of 100µL diluted streptavidin-HRP was added to all wells, including the blank wells.
8. An adhesive film was placed over the plate and incubated at RT for 1 hour on the Labnet Orbit 300 laboratory shaker set at 400rpm.
9. The adhesive film was removed and the wells were emptied. The microwell strips were washed 4 times according to step 1 in the procedure.
10. A volume of 100µL 3,3’,5,5’-Tetramethylbenzidine (TMB) substrate solution was added to all wells.
11. The microwell strips were incubated at RT for 10 minutes in the dark. The optical density of each well was determined at 450 and 620nm within 30 minutes using the VersaMax Microplate reader from Molecular Devices with SoftMax Pro 6.2.2. Data generated was analysed using the 5-parameter curve fit setting. The resultant constants for the cubic equation were entered into a spreadsheet to assess the concentration of IL-6 of the unknown samples on each plate.

2.4.3 Affymetrix eBioscience human IFN-γ ELISA kit

Concentrations of IFN-γ from serum and SF samples, respectively, were determined using a precoated ELISA kit (Affymetrix eBioscience human IFN-γ platinum precoated ELISA kit, catalogue #: BMS228). All the reagents and working standards were prepared according to the procedures described in the technical sheet provided (Appendix V). The laboratory procedure for the IFN-γ ELISA was as follows:

1. The microwell strips were washed twice with approximately 400µL wash buffer per well with thorough aspiration between washes. The wash buffer was allowed to sit in the wells for 10 to 15 seconds before aspiration. This was removed by inversion with gentle tapping. This was followed by blotting the plate against paper towels to remove excess solution.
2. Standard dilutions were prepared on the microwell plate. A volume of 100µL sample diluent was added in duplicate to all standard wells. A volume of 100µL prepared standard (concentration of 200pg.mL^{-1}) was added in duplicate into wells A1 and A2. The contents were mixed with repeated aspiration ejection using a micropipette, and 100µL was transferred to wells B1 and B2, respectively. This was repeated 5 times, creating 2 rows of human IFN-γ standard dilutions, ranging from 100 to 1.56pg.mL^{-1}. A volume of 100µL of the last dilution in the series (G1, G2) was discarded from the last microwells. 100µL of sample diluent was added to H1 and H2 which served as the plate blank.
3. For the serum samples procedure, 50µL of sample diluent was added to all the sample wells. A volume of 50µL of each sample was then added in duplicate to the sample wells. For the SF samples ELISA procedure, 100µL of the hyaluronidase treated samples was added to the wells in duplicate. There were 2 dilutions tested for SF (1:2 and 1:6 dilutions).
4. The biotin conjugate was prepared by adding 60µL of this solution to 5.94mL of 1X assay buffer. A volume of 50µL biotin conjugate was added to all wells. The plate was covered with adhesive film and incubated at RT for 2 hours on the Labnet Orbit 300 laboratory shaker set at 400rpm.
5. The streptavidin-HRP solution was prepared by adding 120µL of streptavidin-HRP to 11.88mL of 1X assay buffer.

6. The adhesive film was removed from the plate and the wells were emptied. The microwell strips were washed according to step 1 of the procedure.
7. A volume of 100µL diluted streptavidin-HRP was added to all wells, including the blank wells.
8. An adhesive film was placed over the plate and incubated at RT for 1 hour on the Labnet Orbit 300 laboratory shaker set at 400rpm.
9. The adhesive film was removed and the wells were emptied. The microwell strips were washed 4 times according to step 1 in the procedure.
10. A volume of 100µL TMB substrate solution was added to all wells.
11. The microwell strips were incubated at RT for 10 minutes in the dark. The optical density of each well was determined at 450 and 620nm within 30 minutes using the VersaMax microplate reader from Molecular Devices with SoftMax Pro 6.2.2. Data generated was analysed using the 5-parameter curve fit setting. The resultant constants for the cubic equation were entered into a spreadsheet to assess the concentration of IFN-γ of the unknown samples on each plate.

2.4.4 Affymetrix eBioscience human IL-13 ELISA kit

Concentrations of IL-13 from serum and SF samples, respectively, were determined using a precoated ELISA kit (Affymetrix eBioscience human IL-13 platinum precoated ELISA kit, catalogue #: BMS231/3). All the reagents and working standards were prepared according to the procedures described in the technical sheet provided (Appendix VI). The laboratory procedure for the IL-13 ELISA was as follows:

1. The microwell strips were washed twice with approximately 400µL wash buffer per well with thorough aspiration between washes. The wash buffer was allowed to sit in the wells for 10 to 15 seconds before aspiration. This was removed by inversion with gentle tapping. This was followed by blotting the plate against paper towels to remove excess solution.
2. The standard dilution of the IL-13 standard was prepared on the microwell plate. A volume of 100µL of 1X assay buffer was added in duplicate to all standard wells. A volume of 100µL prepared standard (200pg.mL^{-1}) was added to wells A1 and A2. The contents were mixed with repeated

aspiration and ejection, and 100µL was transferred to wells B1 and B2, respectively. This was repeated 5 times, creating 2 rows of human IL-13 standard dilutions, ranging from 100 to 1.56pg.mL^{-1}. A volume of 100µL of the last dilution in the series (G1, G2) was discarded from the last microwells. 100µL of 1X assay buffer was added in duplicate to H1 and H2 which served as the plate blank.

3. For the serum samples procedure, 50µL of 1X assay buffer was added to all the sample wells. A volume of 50µL of each sample was then added in duplicate to the sample wells. For the SF samples procedure, 100µL of the hyaluronidase treated samples was added in duplicate.
4. The conjugate mixture was prepared by adding 60µL of conjugate mixture and 120µL of streptavidin-HRP to 5.82mL 1X assay buffer.
5. A volume of 50µL conjugate mixture was added to all wells.
6. The plate was covered with adhesive film and incubated at RT for 2 hours on the Labnet Orbit 300 laboratory shaker set at 500rpm.
7. The adhesive film was removed and the wells were emptied. The microwell strips were washed 4 times according to step 1 of the procedure.
8. A volume of 100µL TMB substrate solution was added to all wells.
9. The microwell strips were incubated at RT for 10 minutes in the dark. The optical density of each well was determined at 450 and 620nm within 30 minutes using the VersaMax microplate reader from Molecular Devices with SoftMax Pro 6.2.2. Data generated was analysed using the 5-parameter curve fit setting. The resultant constants for the cubic equation were entered into a spreadsheet to assess the concentration of IL-13 of the unknown samples on each plate.

2.4.5 Biolegend human IL-10 ELISA kit

All the reagents and working standards were prepared according to the procedures described in the technical sheet provided (Appendix VII). The laboratory procedure for the IL-10 uncoated ELISA (Biolegend human IL-10 ELISA MAX deluxe kit, catalogue #: 430604) was as follows:

1. One day prior to running the ELISA, the capture antibody was diluted in 1X coating buffer. A volume of 60µL capture antibody was diluted in 11.94mL 1X coating buffer. A volume of 100µL of this capture antibody solution was added to all wells of a 96-well plate. This plate was sealed and incubated at 4^{o}C for 17 hours (overnight).
2. The plate was washed 4 times with 400µL wash buffer per well and this was then blotted on absorbent paper by firmly tapping it upside down.
3. A volume of 200µL 1X assay diluent A was added per well to block non-specific binding and to reduce background interference.
4. The plate was sealed and incubated at RT for 1 hour with shaking at 500rpm on the Labnet Orbit 300 laboratory shaker.
5. The dilution series was prepared. The 130ng.mL^{-1} recombinant human IL-10 vial was reconstituted with 0.2mL of 1X assay diluent A. The 250pg.mL^{-1} top standard was prepared by performing a 1:10 dilution by adding 10µL standard stock solution to 90µL of 1X assay diluent A. A volume of 19.23µL of this diluted standard was added to 980.77µL 1X assay diluent A (250pg.mL^{-1}). Six two-fold serial dilutions of the 250pg.mL^{-1} top standard were performed with assay diluent A in separate tubes. The standard concentrations were 250pg.mL^{-1}, 125pg.mL^{-1}, 62.5pg.mL^{-1}, 31.3pg.mL^{-1}, 15.6pg.mL^{-1}, 7.8pg.mL^{-1}, and 3.9pg.mL^{-1}, respectively. The 1X assay diluent A served as the standard group blank.
6. The plate was washed 4 times with wash buffer according to step 2 of the protocol.
7. A volume of 100µL of standards and samples was added to the appropriate wells. For the serum and plasma samples procedure, 100µL of neat serum and/ or plasma from healthy volunteers and patients was added to sample wells in triplicate. For the SF samples procedure, 100µL of the hyaluronidase treated samples was added in triplicate (2-fold dilutions of SF was tested).

8. The plate was sealed and incubated at RT for 2 hours with shaking.
9. The plate was washed 4 times with wash buffer according to step 2 of the protocol.
10. For each plate, a volume of 60µL detection antibody was diluted in 11.94mL 1X assay diluent A. A volume of 100µL diluted detection antibody solution was added to each well, the plate was sealed and incubated at RT for 1 hour with shaking.
11. The plate was washed 4 times with wash buffer according to step 2 of the protocol.
12. For each plate, 12µL avidin-HRP was diluted in 11.99mL assay diluent A. A volume of 100µL diluted avidin-HRP solution was added to each well, the plate was sealed and incubated at RT for 30 minutes with shaking.
13. The plate was washed 5 times with wash buffer according to step 2 of the protocol. For this final wash, wells were soaked in wash buffer for 60 seconds for each wash.
14. For each plate, 6mL of substrate solution A and 6mL of substrate solution B were mixed together. A volume of 100µL freshly mixed TMB substrate solution was added to each well and incubated in the dark for 30 minutes.
15. The reaction was stopped by adding 100µL of stop solution to each well.
10. The optical density of each well was determined at 450 and 570nm within 30 minutes using the VersaMax microplate reader from Molecular Devices with SoftMax Pro 6.2.2. Data generated was analysed using the 5-parameter curve fit setting. The resultant constants for the cubic equation were entered into a spreadsheet to assess the concentration of IL-10 of the unknown samples on each plate.

2.4.6 Biolegend human TNF-α ELISA kit

All the reagents and working standards were prepared according to the procedures described in the technical sheet provided (Appendix VIII). The laboratory procedure for the TNF-α uncoated ELISA (Biolegend human TNF-α ELISA MAX deluxe kit, catalogue #: 430204) was as follows:

1. One day prior to running the ELISA, the capture antibody was diluted in 1X coating buffer. A volume of 60µL capture antibody was diluted in 11.94mL 1X coating buffer and 100µL of this capture antibody solution was added to all wells of a 96-well plate. This plate was sealed and incubated at 4^{o}C for 17 hours (overnight).
2. The plate was washed 4 times with 400µL wash buffer per well and this was then blotted on absorbent paper by firmly tapping it upside down.
3. A volume of 200µL 1X assay diluent A was added per well to block non-specific binding and to reduce background interference.
4. The plate was sealed and incubated at RT for 1 hour with shaking at 200rpm on a Labnet Orbit 300 laboratory shaker.
5. The dilution series was prepared. The TNF-α standard was reconstituted with 0.2mL 1X assay diluent A and had a concentration of 40ng.mL^{-1}. The 500pg.mL^{-1} top standard was prepared by adding 12.5µL of the reconstituted standard to 987.5µL of 1X assay diluent A. Six 2-fold dilutions of this standard was performed using 1X assay diluent A to make the series dilution (500pg.mL^{-1}, 250pg.mL^{-1}, 125pg.mL^{-1}, 62.5pg.mL^{-1}, 31.3pg.mL^{-1}, 15.6pg.mL^{-1}, 7.8pg.mL^{-1}).The 1X assay diluent A served as the zero standard and blank.
6. The plate was washed 4 times with wash buffer according to step 2 of the protocol.
7. A volume of 100µL of standards and samples was added to the appropriate wells. For the serum and plasma samples procedure, 100µL of neat serum and/ or plasma from healthy volunteers and patients was added to sample wells in triplicate where applicable, and in duplicate where limited sample was available. For the SF samples procedure, 100µL of the hyaluronidase treated samples was added in duplicate. The 2 dilutions tested were 1:2 and 1:16. The plate was sealed and incubated at RT for 2 hours with shaking.

8. The plate was washed 4 times with wash buffer according to step 2 of the protocol.
9. For each plate, 60µL detection antibody was diluted in 11.94mL 1X assay diluent A. A volume of 100µL diluted detection antibody solution was added to each well, the plate was sealed and incubated at RT for 1 hour with shaking.
10. The plate was washed 4 times with wash buffer according to step 2 of the protocol.
11. For each plate, 12µL avidin-HRP was diluted in 11.99mL assay diluent A. A volume of 100µL diluted avidin-HRP solution was added to each well, the plate was sealed and incubated at RT for 30 minutes with shaking.
12. The plate was washed 5 times with wash buffer according to step 2 of the procedure. For this final wash, wells were soaked in wash buffer for 60 seconds for each wash.
13. For each plate, 6mL of substrate solution A and 6mL of substrate solution B were mixed together. 100µL of freshly mixed TMB substrate solution was added to each well and incubated in the dark for 15 minutes.
14. The reaction was stopped by adding 100µL of stop solution to each well.
16. The optical density of each well was determined at 450 and 570nm within 30 minutes using the VersaMax microplate reader from Molecular Devices with SoftMax Pro 6.2.2. Data generated was analysed using the 5-parameter curve fit setting. The resultant constants for the cubic equation were entered into a spreadsheet to assess the concentration of TNF-α of the unknown samples on each plate.

2.4.7 Biolegend human IL-17A ELISA kit

All the reagents and working standards were prepared according to the procedures described in the technical sheet provided (Appendix IX). The laboratory procedure for the IL-17A uncoated ELISA (Biolegend human IL-17A ELISA MAX deluxe kit, catalogue #: 433914) was as follows:

1. One day prior to running the ELISA, the capture antibody was diluted in 1X coating buffer. A volume of 60µL capture antibody was diluted in 11.94mL 1X coating buffer and 100µL of this capture antibody solution was added to all wells of a 96-well plate. This plate was sealed and incubated at 4^{o}C for 17 hours (overnight).
2. The plate was washed 4 times with 400µL wash buffer per well and this was then blotted on absorbent paper by firmly tapping it upside down.
3. A volume of 200µL 1X assay diluent A was added per well to block non-specific binding and to reduce background interference.
4. The plate was sealed and incubated at RT for 1 hour with shaking at 200rpm on the Labnet Orbit 300 laboratory shaker.
5. The dilution series was prepared. The IL-17A standard was reconstituted with 0.2mL of 1X assay diluent A (concentration of 65ng.ml^{-1}). The 250pg.mL^{-1} top standard was prepared by adding 3.8µL of the reconstituted standard to 996.2µL of 1X assay diluent A. Six 2-fold dilutions of this standard was performed using 1X assay diluent A to make the series dilution (250pg.mL^{-1}, 125pg.mL^{-1}, 62.5pg.mL^{-1}, 31.3pg.mL^{-1}, 15.6pg.mL^{-1}, 7.8pg.mL^{-1}, 3.9pg.mL^{-1}). 1X assay diluent A served as the zero standard and blank.
6. The plate washed 4 times with wash buffer according to step 2 of the protocol.
7. A volume of 100µL of standards and samples were added to the appropriate wells. For the serum and plasma samples procedure, 100µL of neat serum and/ or plasma from healthy volunteers and patients was added to sample wells in triplicate where applicable, and in duplicate where limited sample was available. For the SF samples procedure, 100µL of the hyaluronidase treated samples was added in triplicate. The plate was sealed and incubated for 2 hours with shaking.

8. The plate was washed 4 times with wash buffer according to step 2 of the protocol.
9. For each plate, 60µL detection antibody was diluted in 11.94mL 1X assay diluent A. A volume of 100µL diluted detection antibody solution was added to each well, the plate was sealed and incubated at RT for 1 hour with shaking.
10. The plate was washed 4 times with wash buffer according to step 2 of the protocol.
11. For each plate, 12µL avidin-HRP was diluted in 11.99mL assay diluent A. A volume of 100µL diluted avidin-HRP solution was added to each well, the plate was sealed and incubated at RT for 30 minutes with shaking.
12. The plate was washed 5 times with wash buffer according to step 2 of the protocol. For this final wash, wells were soaked in wash buffer for 60 seconds for each wash.
13. For each plate, 6mL of substrate solution A and 6mL of substrate solution B were mixed together. A volume of 100µL freshly mixed TMB substrate solution was added to each well and incubated in the dark for 30 minutes.
14. The reaction was stopped by adding 100µL of stop solution to each well.
15. The optical density of each well was determined at 450 and 570nm within 30 minutes using the VersaMax microplate reader from Molecular Devices with SoftMax Pro 6.2.2. Data generated was analysed using the 5-parameter curve fit setting. The resultant constants for the cubic equation were entered into a spreadsheet to assess the concentration of IL-17A of the unknown samples on each plate.

2.5 Statistical analysis

All individual mean cytokine concentrations for patient and healthy volunteers in the study were plotted on histograms using Microsoft Excel version 2010 (Microsoft Windows and Office). Data analyses were performed using IBM® SPSS® statistics version 22.0 [Copyright IBM Corporation and other(s) 1989, 2013]. All the descriptive statistics for cytokine analysis were presented as the mean ± standard deviation (mean ± SD). Using SPSS, boxplots were generated to determine if outliers were present in the serum data. The Shapiro-Wilk's test for normality was conducted to determine if data was normally distributed, where $P<0.05$. The non-parametric Mann Whitney *U* test was conducted to determine if there was a significant difference in cytokine concentrations between the patient and healthy volunteer groups, where $P<0.05$. A paired samples *t*-test was conducted to compare IL-6 serum mean concentrations in healthy volunteers ($n=9$) at time-point "a" and time-point "b" in the study.

Chapter 3: Results

3.1 IL-6 concentrations in the study groups with statistical analysis

The IL-6 data was presented using histograms based on the ELISA kit used to test the serum and SF samples and the sample type tested (Figure 3.1). The total number of participants for the analysis of serum IL-6 in the current study was 42 (24 patients and 18 healthy volunteers) where the mean ± SD concentration determined for the patient group was 4.78 ± 4.63pg.mL^{-1} and 1.66 ± 0.64pg.mL^{-1} for the healthy volunteer group. IL-6 was elevated in the SF of many of the patients in the study (Figure 3.3). The mean IL-6 SF concentration in the patient group ranged from 13.19pg.mL^{-1} to 2258.39pg.mL^{-1}. The mean ± SD IL-6 concentration in SF was 417.91 ± 658.21pg.mL^{-1}, where 18 patients participated (P1 to P9, P11 to P15, P23, P26, P30 and P32). P10 had a dry knee so no SF was available for analysis. In matched serum and SF from the patients tested, the SF concentration of IL-6 was higher than their matched serum sample (Figure 3.3).

For serum IL-6, there were outliers present in the data, as assessed by inspection of a boxplot (Figure 3.4). Medians for each group were 2.88pg.mL^{-1} for the patient group and 1.59pg.mL^{-1} for the healthy volunteer group. The IL-6 concentrations in the patient group were not normally distributed, as assessed by Shapiro-Wilk's test (P<0.05). The IL-6 concentrations in the healthy volunteer group were normally distributed, P>0.05. A Mann-Whitney U test was conducted to determine if there were differences in IL-6 concentrations between the patient and healthy volunteer groups. Distributions of IL-6 concentrations in the patient and healthy volunteer groups were not similar, as assessed by visual inspection. There was a statistically difference in mean IL-6 concentrations between the patient group (mean rank= 27.67pg.mL^{-1}) and healthy volunteer group (mean rank= 13.28pg.mL^{-1}), U= 68, z= -3.762, P<0.05 (where U, the "Mann-Whitney U" value; z, z-score which is the "Standardised test statistic" value).

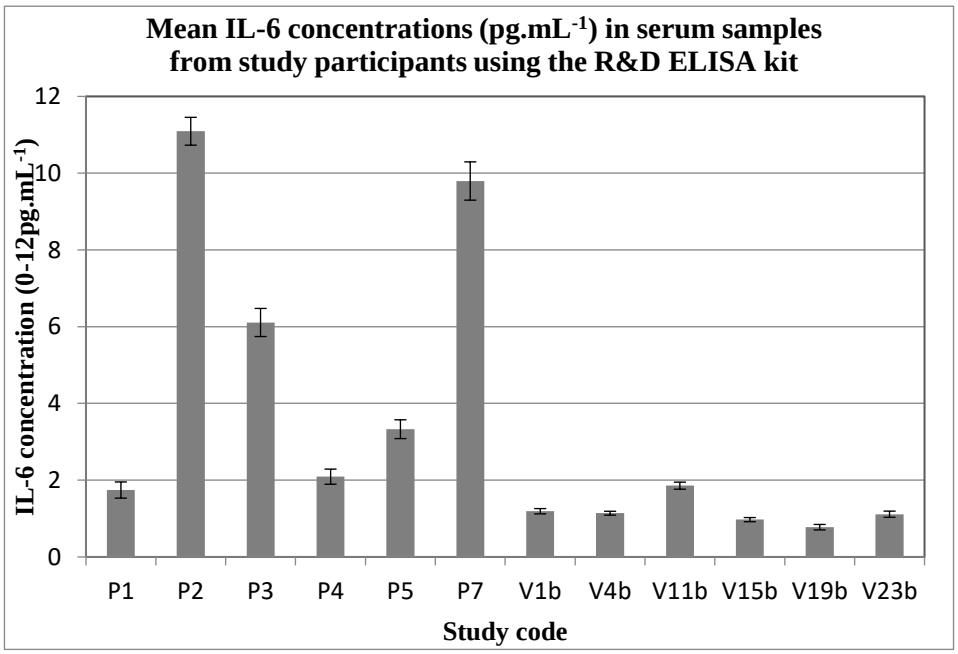
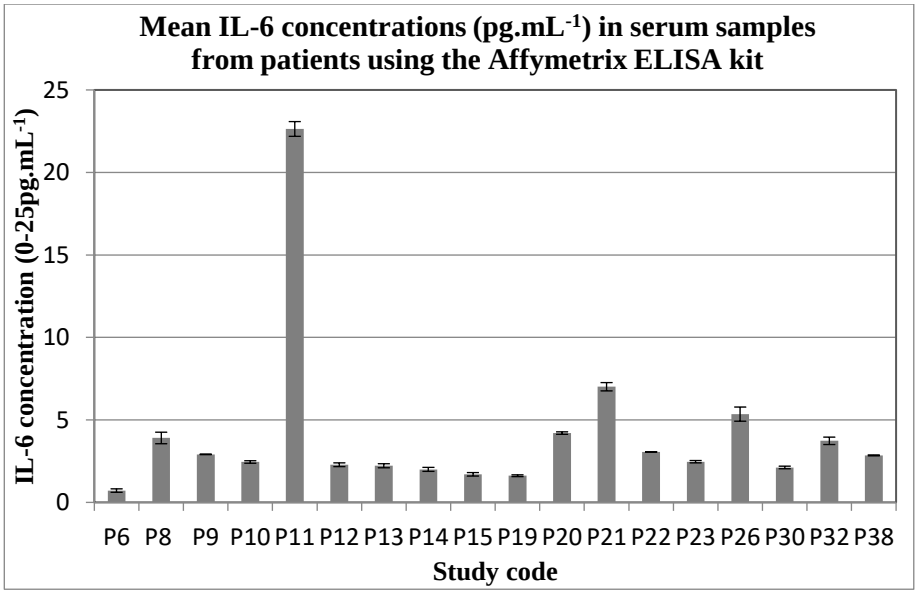
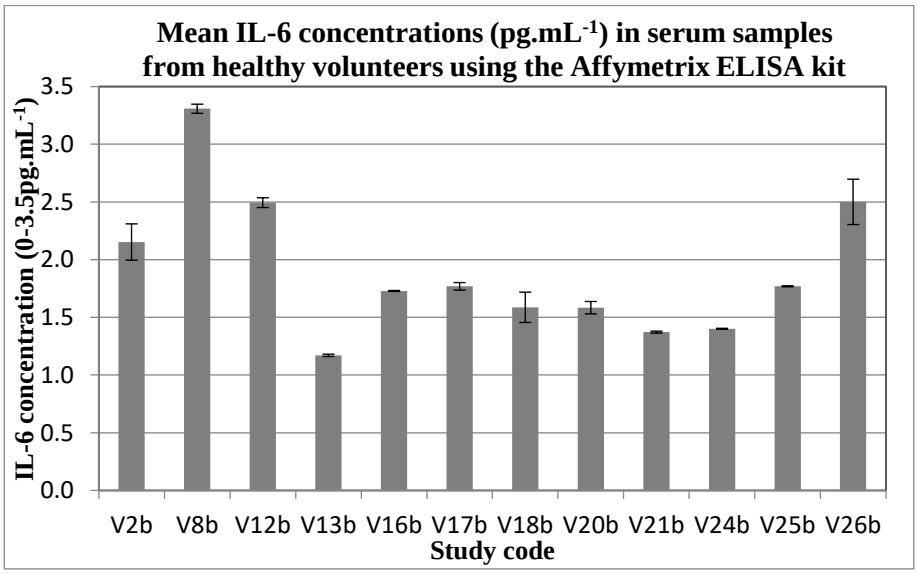


Figure 3.1: Mean IL-6 concentrations (pg.mL^{-1}) in serum samples from study participants using the R&D systems ELISA kit. All serum samples from patients and healthy volunteers were tested in triplicate using the R&D Systems precoated IL-6 ELISA kit (catalogue #: D6050). Error bars were calculated using the SD of the sample triplicate replicates. The 5-parameter curve fit was used for data analysis (n=12, 6 patients and 6 healthy volunteers). ELISA, enzyme linked immunosorbent assay; IL-6, interleukin-6; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.



3.2a. Mean IL-6 concentrations in serum from patients (error bars denote SD).



3.2b. Mean IL-6 concentrations in serum from healthy volunteers (error bars denote SD).

Figure 3.2a: Mean IL-6 concentrations (pg.mL^{-1}) in serum from patients; 3.2b: Mean IL-6 concentrations (pg.mL^{-1}) in serum from healthy volunteers. All samples were tested in duplicate using the IL-6 Affymetrix eBiosciences ELISA kit (catalogue #:BMS213/2CE). Error bars were calculated using the SD of sample duplicate replicates (n=30, 18 patients and 12 healthy volunteers). ELISA, enzyme linked immunosorbent assay; IL-6, interleukin-6; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

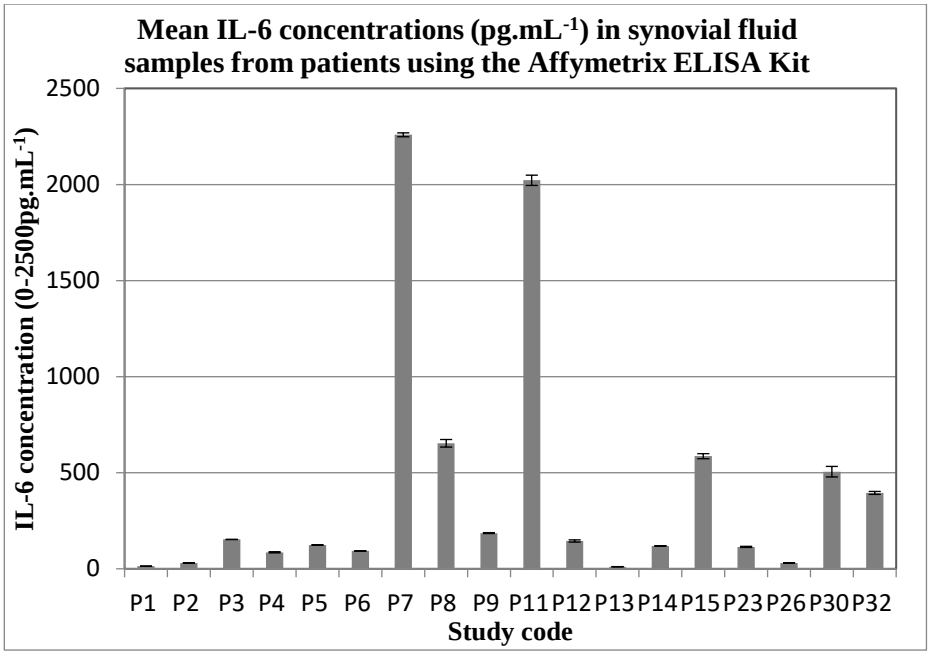


Figure 3.3: Mean IL-6 concentrations (pg.mL^{-1}) in synovial fluid samples from patients using the Affymetrix ELISA kit (n=18). All samples were tested in duplicate using the IL-6 Affymetrix eBiosciences ELISA kit (catalogue #:BMS213/2CE). Error bars were calculated using the SD of the sample duplicate replicates. ELISA, enzyme linked immunosorbent assay; IL-6, interleukin-6; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

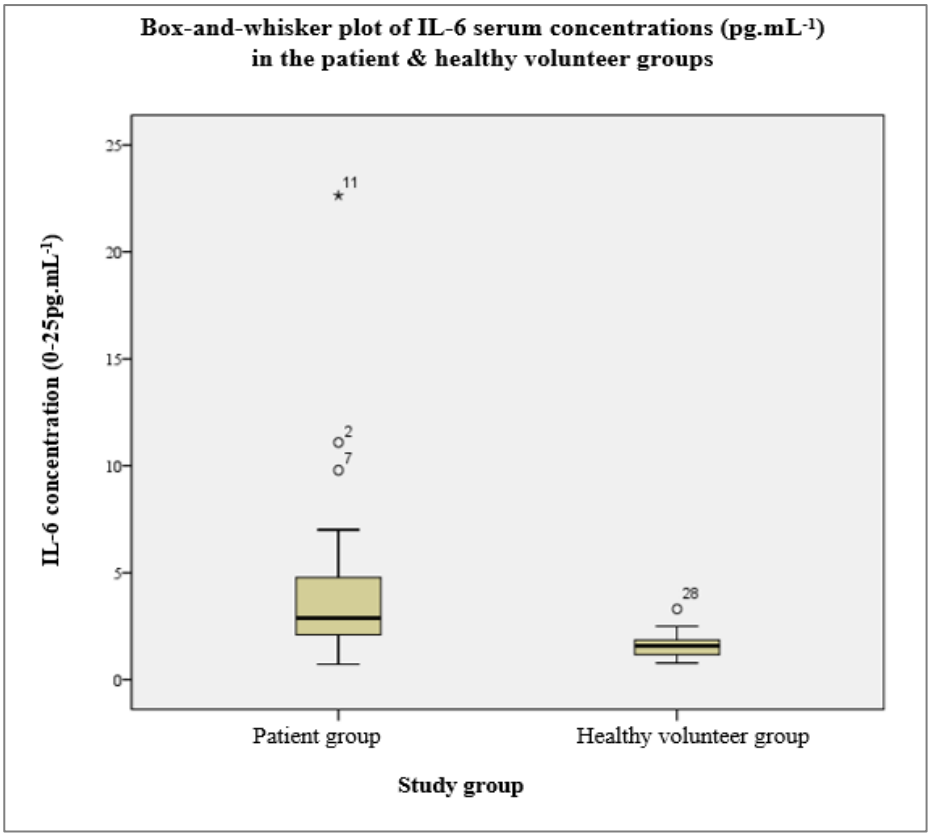
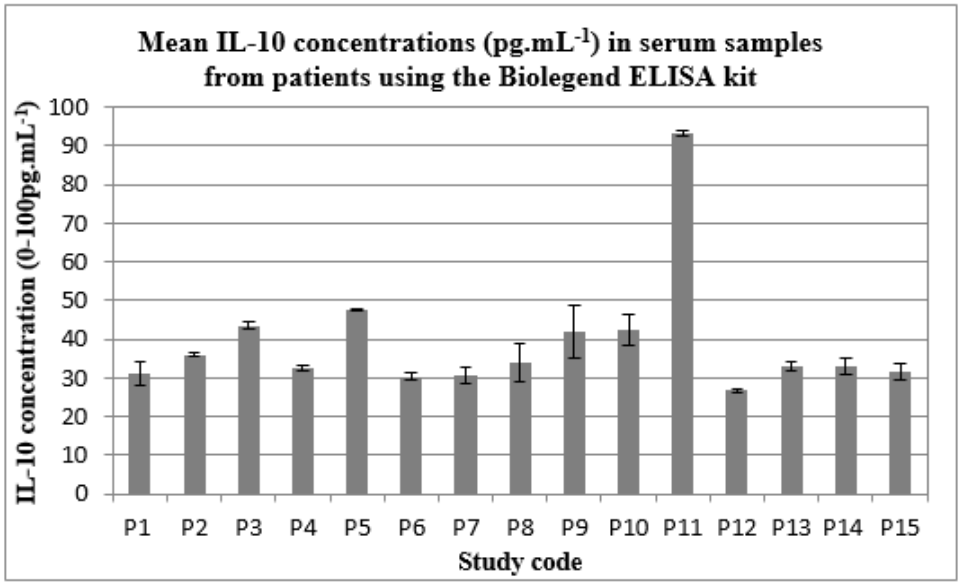


Figure 3.4: Box-and-whisker plot of IL-6 serum concentrations (pg.mL^{-1}) in the patient and healthy volunteer groups (n=42, 24 patients and 18 healthy volunteers). The boxplot gave a visual depiction of the distribution of data in both groups tested with means of serum replicates plotted. Boxes represent the interquartile range (25th to 75th percentile) and the line across the box indicates the median. Outliers were observed in both groups; circular dots were outliers, while extreme outliers were indicated with an asterix. IL-6, interleukin-6; n, number of subjects.

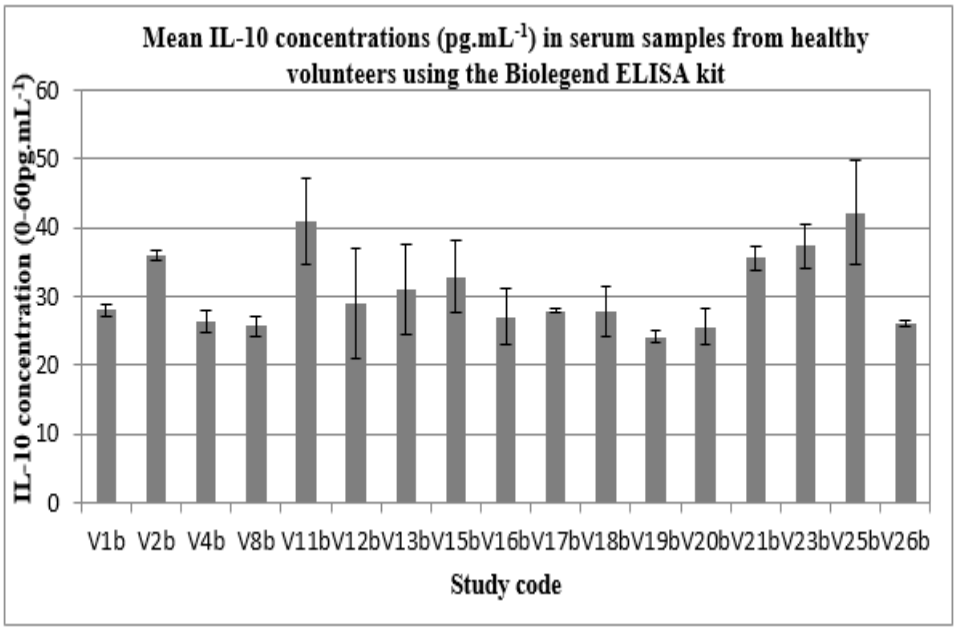
3.2 IL-10 concentrations in the study groups with statistical analysis

The IL-10 data was presented using histograms based on the specimen type tested, either serum or SF (Figure 3.5 and Figure 3.6, respectively). The Biolegend IL-10 ELISA MAX Deluxe uncoated kit (catalogue #: 430604) was used for all IL-10 cytokine quantification. The total number of participants for the analysis of serum IL-10 in the current study was 32 (15 patients and 17 healthy volunteers) where the mean ± SD concentration determined for the patient group was 39.17 ± 16.09pg.mL^{-1} and 30.80 ± 5.66pg.mL^{-1} for the healthy volunteer group. Only 3 patients had detectable concentrations of IL-10 in the SF samples tested (Figure 3.6). The SF concentration of IL-10 was low in many patients with concentrations below the limit of detection for the ELISA kit used (Figure 3.6), where 18 patients participated (P1 to P9, P11 to P15, P23, P26, P30 and P32). P10 had a dry knee so no SF was available for analysis.

For serum IL-10, there were outliers present in the data, as assessed by inspection of a boxplot (Figure 3.7). Medians for each group were 33.03pg.mL^{-1} for the patient group and 27.99pg.mL^{-1} for the healthy volunteer group. The IL-10 concentrations in the patient and healthy volunteer groups were not normally distributed, as assessed by Shapiro-Wilk's test (P<0.05). A Mann-Whitney U test was conducted to determine if there were differences in median IL-10 concentrations between the patient and the healthy volunteer group. Distributions of IL-10 concentrations in the patient and healthy volunteer groups were similar, as assessed by visual inspection. Median IL-10 concentrations was significantly higher in the patient group (33.03pg.mL^{-1}) than the healthy volunteer group (27.99pg.mL^{-1}), U= 64, z= -2.398, P= 0.016 (where U, the "Mann-Whitney U" value; z, z-score which is the "standardised test statistic" value).



3.5a. Mean IL-10 concentrations in serum from patients (error bars denote SD).



3.5b. Mean IL-10 concentrations in serum from healthy volunteers (error bars denote SD).

Figure 3.5a: Mean IL-10 concentrations (pg.mL^{-1}) in serum from patients; 3.5b. Mean IL-10 concentrations (pg.mL^{-1}) in serum from healthy volunteers. All samples were tested in triplicate using the Biolegend Human IL-10 ELISA MAX Deluxe kit (catalogue #:430604). Error bars were calculated using the SD of sample triplicate replicates (n=32, 15 patients, 17 healthy volunteers). ELISA, enzyme linked immunosorbent assay; IL-10, interleukin-10; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

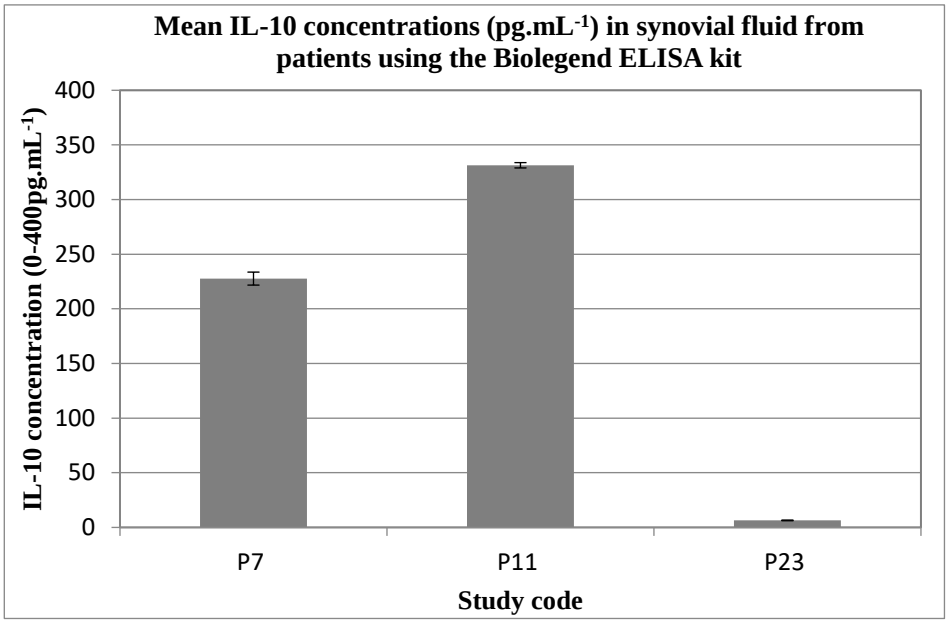


Figure 3.6: Mean IL-10 concentrations (pg.mL^{-1}) in synovial fluid samples from patients using the Biolegend ELISA kit (n=15). All samples were tested in triplicate using the using the Biolegend human IL-10 ELISA MAX Deluxe kit (catalogue #:430604). Only P7, P11 and P23 had detectable concentrations of IL-10. Many patients tested had undetectable concentrations of IL-10 which included P1 to P6, P8, P9, P12 to 15, and P23. Error bars were calculated using the SD of the sample triplicate replicates. ELISA, enzyme linked immunosorbent assay; IL-10, interleukin-10; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

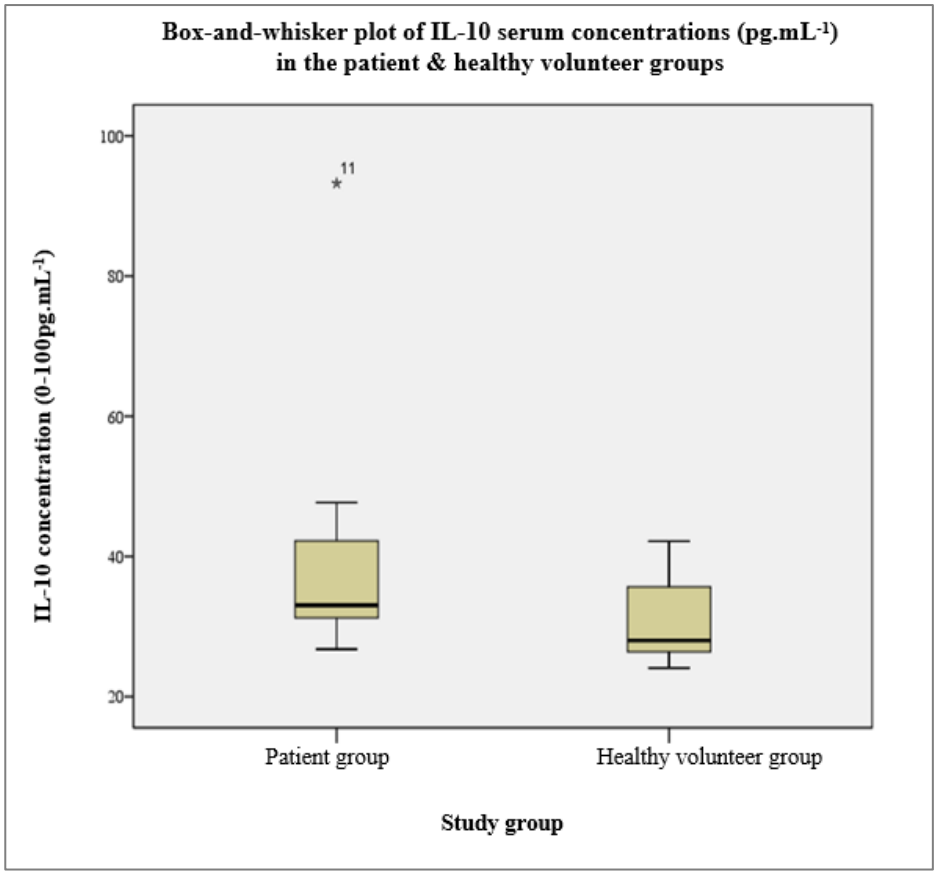


Figure 3.7: Box-and-whisker plot of IL-10 serum concentrations (pg.mL^{-1}) in the patient and healthy volunteer groups (n=32, 15 patients and 17 healthy volunteers). The boxplot gave a visual depiction of the distribution of data in both groups tested with means of serum replicates plotted. Boxes represent the interquartile range (25th to 75th percentile) and the line across the box indicates the median. An outlier was observed in the patient group. This was an extreme outlier which was indicated with an asterix. IL-10, interleukin-10; n, number of subjects.

3.3 IL-17A concentrations in the study groups with statistical analysis

The IL-17A data was presented using histograms based on the number of replicates for each sample and the specimen type tested, either serum or SF (Figure 3.8; Figure 3.9; and Figure 3.10). Serum samples were either tested in triplicate or duplicate, where availability of sample determined the number of replicates performed. The Biolegend IL-17A ELISA MAX Deluxe uncoated kit (catalogue #: 433914) was used for all IL-17A cytokine quantification. The total number of participants for the analysis of serum IL-17A in the current study was 38 (20 patients and 18 healthy volunteers) where the mean ± SD concentration determined for the patient group was 8.33 ± 3.81pg.mL^{-1} and 9.16 ± 5.33pg.mL^{-1} for the healthy volunteer group. In the healthy volunteer group, the highest mean ± SD serum concentration of IL-17A was 17.15 ± 2.06pg.mL^{-1} (V8b), while the lowest was 3.37 ± 0.05pg.mL^{-1} (V13b) (Figure 3.9b). There were 18 patients (P1 to P9, P11 to P15, P23, P26, P30 and P32) that participated in SF analysis. The SF concentration of IL-17A fluctuated from patient to patient (Figure 3.10) with the highest concentrations seen in 3 patients (P5, P13 and P14). Each of these patients had IL-17A concentrations greater than 20pg.mL^{-1}. P10 had a dry knee so no SF was available for analysis.

For serum IL-17A, there were no outliers in the data, as assessed by inspection of a boxplot for values greater than 1.5 box-lengths from the edge of the box (Figure 3.11). Medians for each group were 9.30pg.mL^{-1} for the patient group and 8.14pg.mL^{-1} for the healthy volunteer group. The IL-17A concentrations in the patient and healthy volunteer groups were not normally distributed, as assessed by Shapiro-Wilk's test (P<0.05). A Mann-Whitney U test was conducted to determine if there were differences in median IL-17A concentrations between the patient and the healthy volunteer group. Distributions of IL-17A concentrations in the patient and healthy volunteer groups were similar, as assessed by visual inspection. Median IL-17A concentrations for the patient group (9.30pg.mL^{-1}) and the volunteer group (8.14pg.mL^{-1}) were not statistically significant, U= 159, z= -0.614, P= 0.553 (where U, the "Mann-Whitney U" value; z, z-score which is the "Standardised test statistic" value).

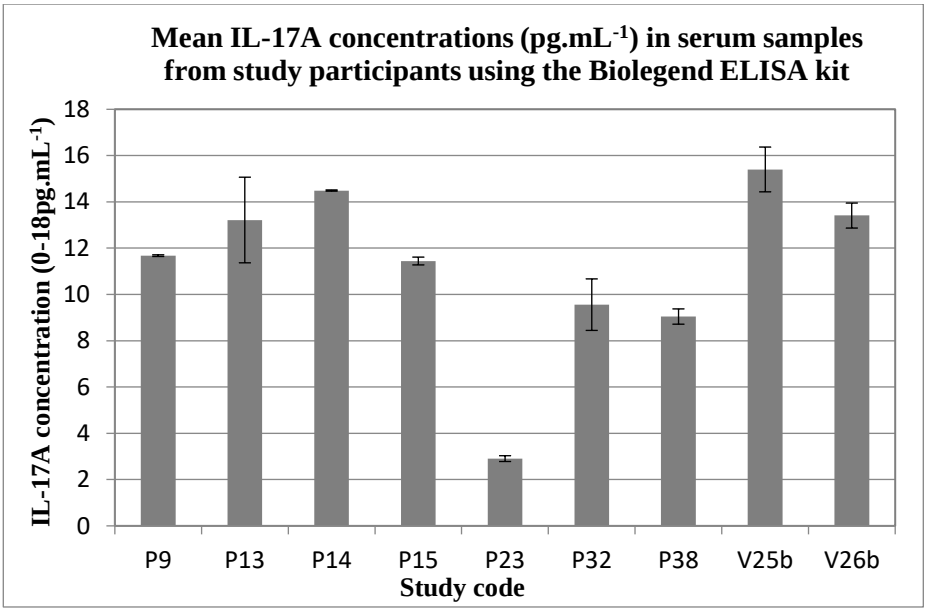
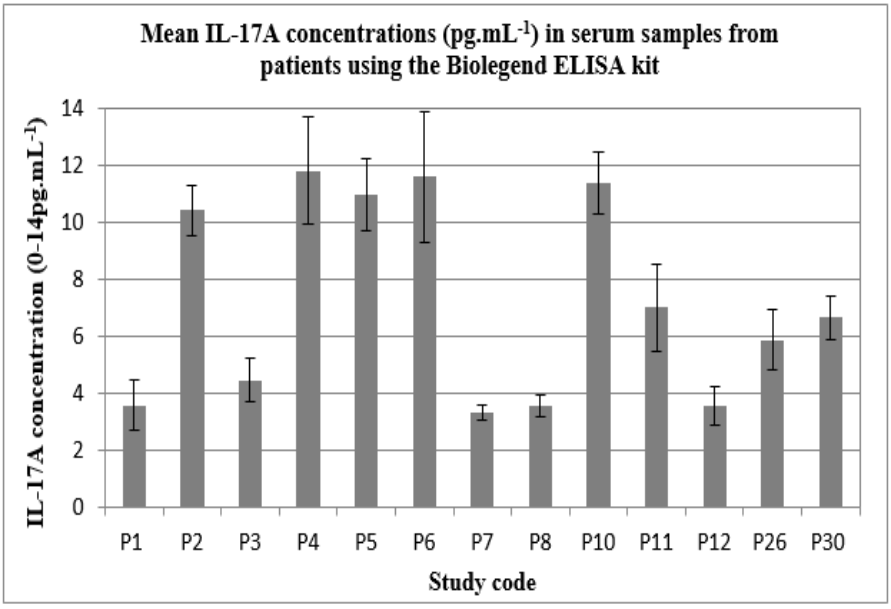
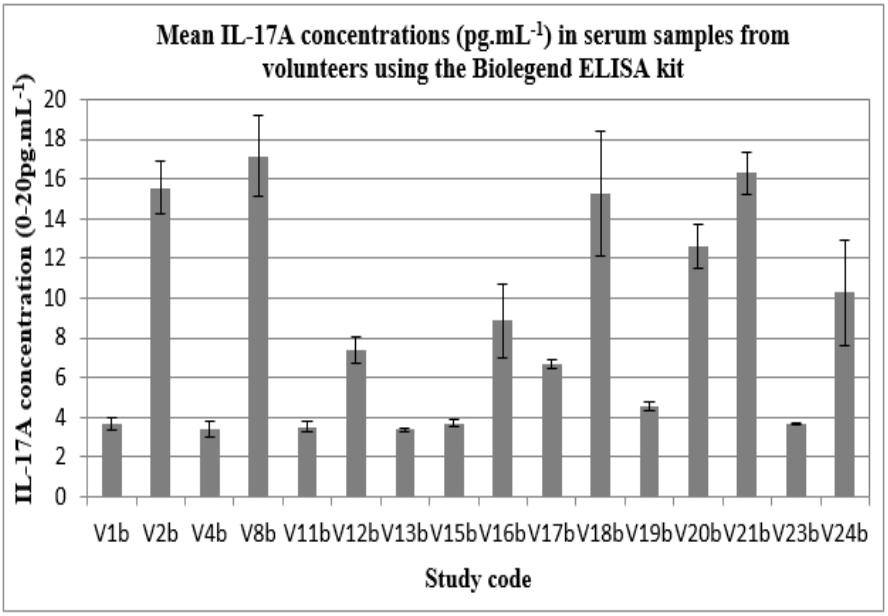


Figure 3.8: Mean IL-17A concentrations (pg.mL^{-1}) in serum samples from study participants using the Biolegend ELISA kit. All serum samples from patients and healthy volunteers were tested in duplicate using the Biolegend human IL-17A ELISA MAX Deluxe kit (catalogue #: 433914). Error bars were calculated using the SD of the sample triplicate replicates. The 5-parameter curve fit was used for data analysis (n=9, 7 patients and 2 healthy volunteers). ELISA, enzyme linked immunosorbent assay; IL-17A, interleukin-17A; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.



3.9a. Mean IL-17A concentrations in serum from patients (error bars denote SD).



3.9b. Mean IL-17A concentrations in serum from healthy volunteers (error bars denote SD).

Figure 3.9a. Mean IL-17A concentrations (pg.mL^{-1}) in serum from patients; 3.9b. Mean IL-17A concentrations (pg.mL^{-1}) in serum from healthy volunteers. All samples were tested in triplicate using the Biolegend Human IL-17A ELISA MAX Deluxe kit (catalogue #:433914). Error bars were calculated using the SD of sample triplicate replicates (n=29, 13 patients and 16 healthy volunteers)]. ELISA, enzyme linked immunosorbent assay; IL-17A, interleukin-17A; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

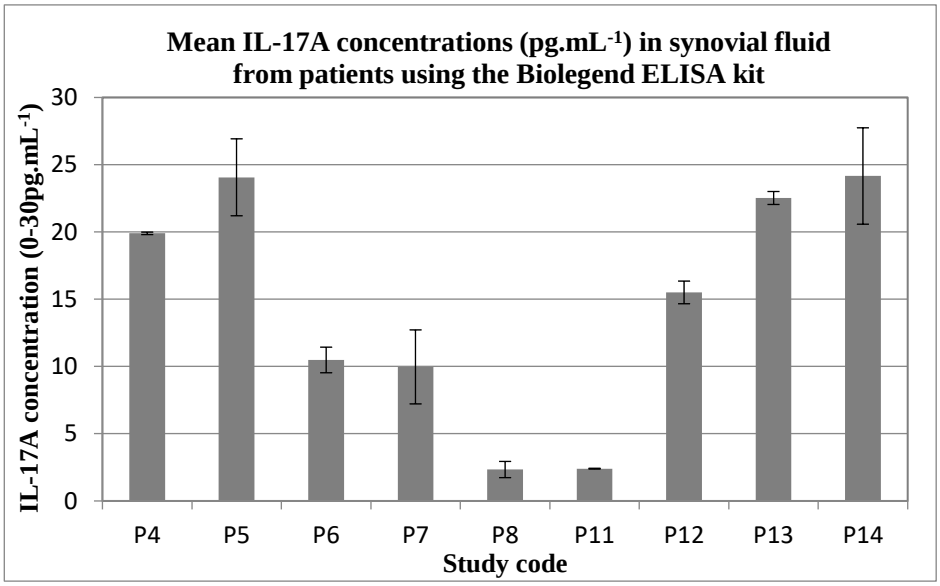


Figure 3.10: Mean IL-17A concentrations (pg.mL^{-1}) in synovial fluid samples from patients using the Biolegend ELISA kit (n=18). Synovial fluid from patients (P4, P8, P11 and P12) were tested in duplicate while all other patients were tested in triplicate (P1 to P3, P9, P13, P14, P15, P23, P26, P30 and P32) using the Biolegend human IL-17A ELISA MAX Deluxe kit (catalogue #:433914). The patients (P1, P2, P3, P9, P15, P23, P26, P30 and P32) had IL-17A concentrations below the limit of detection for the ELISA kit used. Error bars were calculated using the SD of the sample replicates. ELISA, enzyme linked immunosorbent assay; IL-17A, interleukin-17A; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

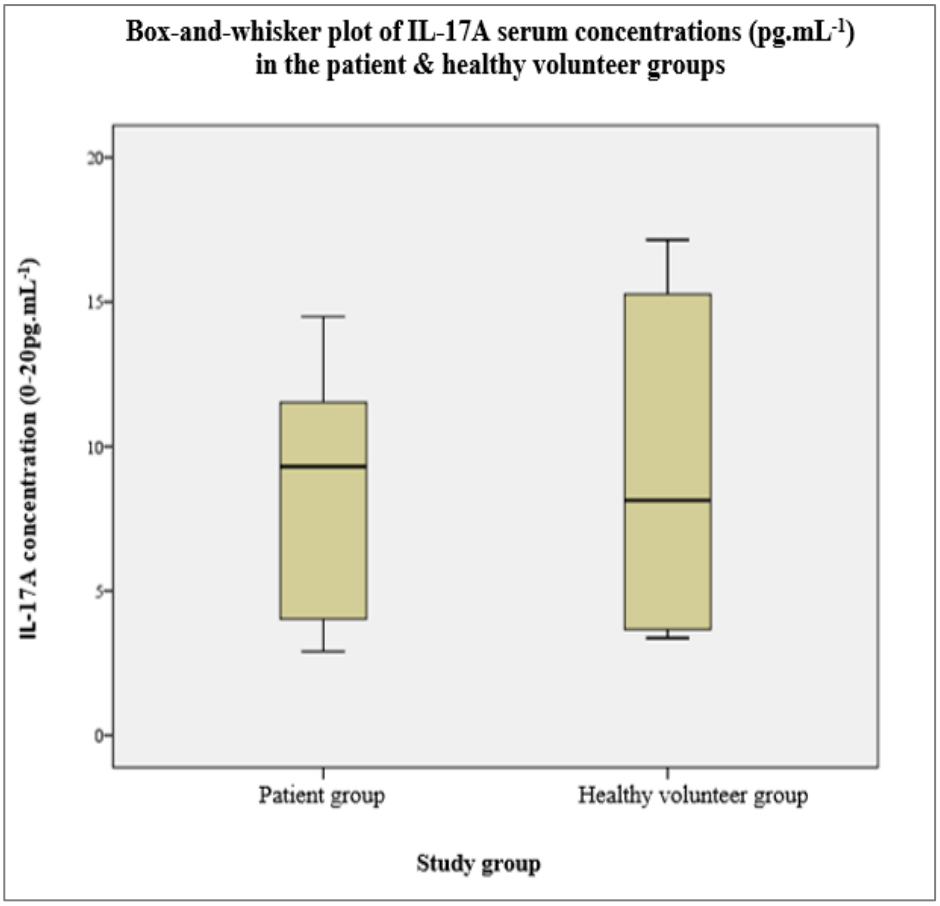


Figure 3.11: Box-and-whisker plot of IL-17A serum concentrations (pg.mL^{-1}) in the patient and healthy volunteer groups (n=38, 20 patients and 18 healthy volunteers). The boxplot gave a visual depiction of the distribution of data in both groups tested with means of serum replicates plotted. Boxes represent the interquartile range (25th to 75th percentile) and the line across the box indicates the median. No outliers were observed in the groups. IL-17A, interleukin-17A; n, number of subjects.

3.4 TNF-α concentrations in the study groups with statistical analysis

The TNF-α data was presented using histograms based on the number of replicates for each sample and the specimen type tested, either serum or SF (Figure 3.12; Figure 3.13; and Figure 3.14). Serum samples were either tested in triplicate or duplicate, where availability of sample determined the number of replicates performed. The Biolegend TNF-α ELISA MAX Deluxe uncoated kit (catalogue #: 430204) was used for all TNF-α cytokine quantification. All serum samples tested in the patient and healthy volunteer groups had detectable concentrations of TNF-α present. The total number of participants for the analysis of serum TNF-α in the current study was 38 (20 patients and 18 healthy volunteers) where the mean ± SD concentration determined for the patient group was 14.68 ± 8.66pg.mL^{-1} and 14.29 ± 6.58pg.mL^{-1} for the healthy volunteer group. The range of mean serum values for TNF-α in the patient group was 5.38 to 39.38pg.mL^{-1}, while the range was 3.50 to 27.94pg.mL^{-1} in the healthy volunteer group. There were 18 patients (P1 to P9, P11 to P15, P23, P26, P30 and P32) that participated in SF analysis. All the participants had undetectable concentrations of TNF-α in SF, except P7 which had a mean ± SD concentration of 25.08 ± 0.91pg.mL^{-1} (samples were tested in duplicate replicates). P10 had a dry knee so no SF was available for analysis.

For serum TNF-α, there were outliers present in the data, as assessed by inspection of a boxplot (Figure 3.14). Medians for each group were 12.22pg.mL^{-1} for the patient group and 12.22pg.mL^{-1} for the healthy volunteer group. The TNF-α concentrations in the patient group were not normally distributed, as assessed by Shapiro-Wilk's test (P<0.05). The TNF-α concentrations in the healthy volunteer group were normally distributed (P>0.05). A Mann-Whitney U test was conducted to determine if there were differences in median TNF-α concentrations between the patient and the healthy volunteer group. Distributions of TNF-α concentrations in the patient and healthy volunteer groups were similar, as assessed by visual inspection. Median TNF-α concentrations for the patient group (12.22pg.mL^{-1}) and the healthy volunteer group (12.58pg.mL^{-1}) was not statistically significant, U= 169, z= -0.322, P= 0.762.

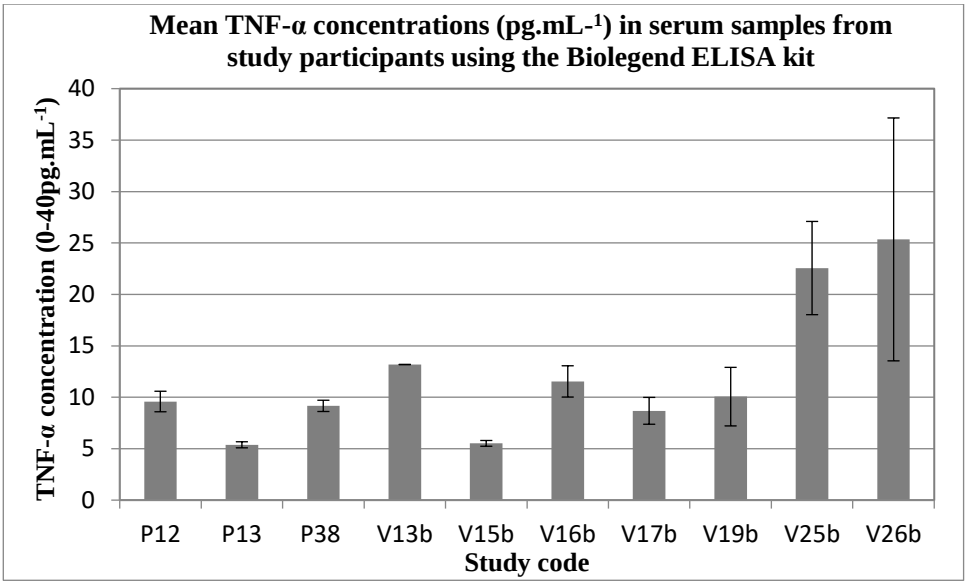
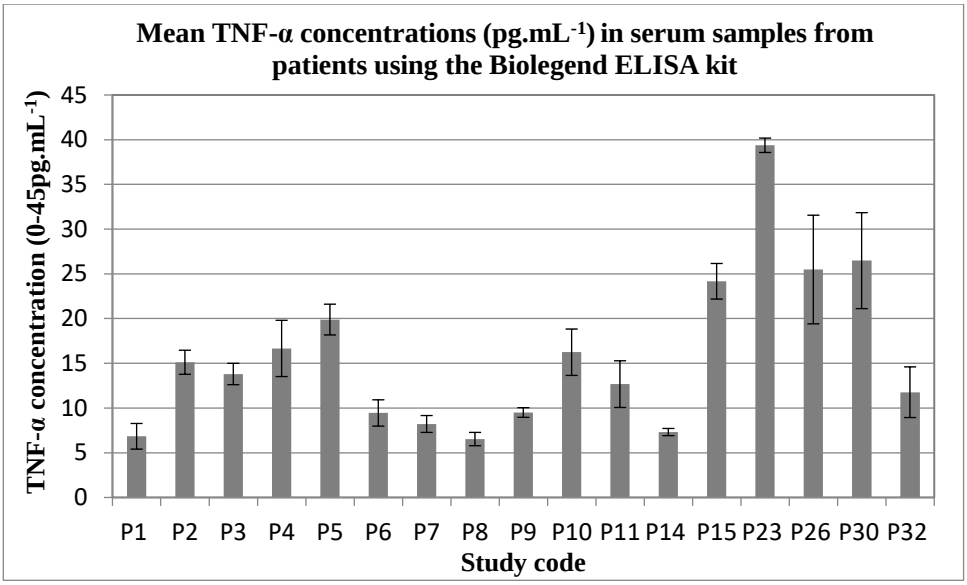
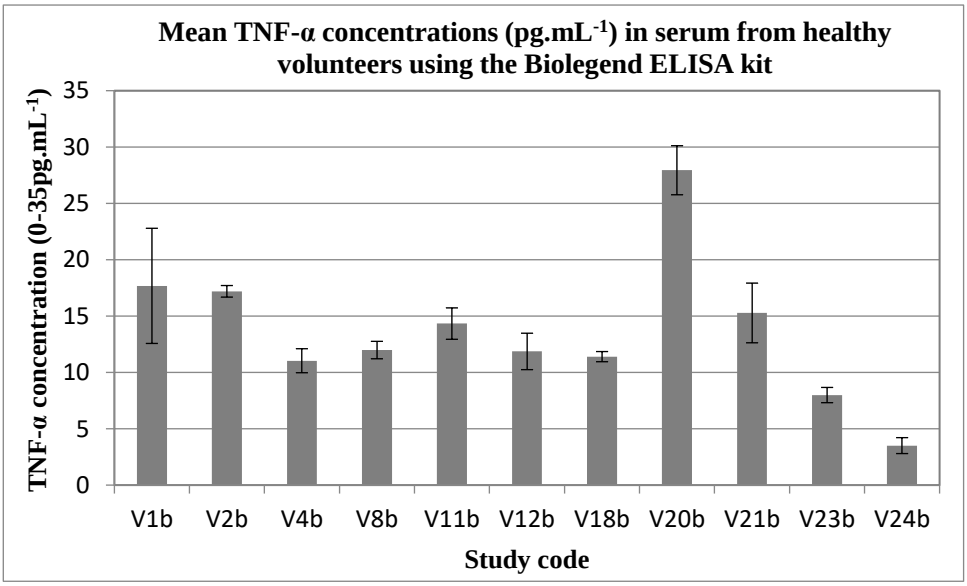


Figure 3.12: Mean TNF-α concentrations (pg.mL^{-1}) in serum samples from study participants using the Biolegend ELISA kit. All serum samples from patients and healthy volunteers were tested in duplicate using the Biolegend human TNF-α ELISA MAX Deluxe kit (catalogue #: 430204). Error bars were calculated using the SD of the sample duplicate replicates. The 5-parameter curve fit was used for data analysis (n=10, 3 patients and 7 healthy volunteers). ELISA, enzyme linked immunosorbent assay; TNF-α, tumour necrosis factor-alpha; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.



3.13a. Mean TNF-α concentrations (pg.mL^{-1}) in serum from patients (error bars denote SD).



3.13b. Mean TNF-α (pg.mL^{-1}) concentrations in serum from healthy volunteers (error bars denote SD).

Figure 3.13a. Mean TNF-α concentrations (pg.mL^{-1}) in serum from patients; 3.13b. Mean TNF-α concentrations (pg.mL^{-1}) in serum from healthy volunteers. All samples were tested in triplicate using the Biolegend Human TNF-α ELISA MAX Deluxe kit (catalogue #: 430204). Error bars were calculated using the SD of sample triplicate replicates (n=28, 17 patients and 11 healthy volunteers). ELISA, enzyme linked immunosorbent assay; TNF-α, tumour necrosis factor-alpha; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

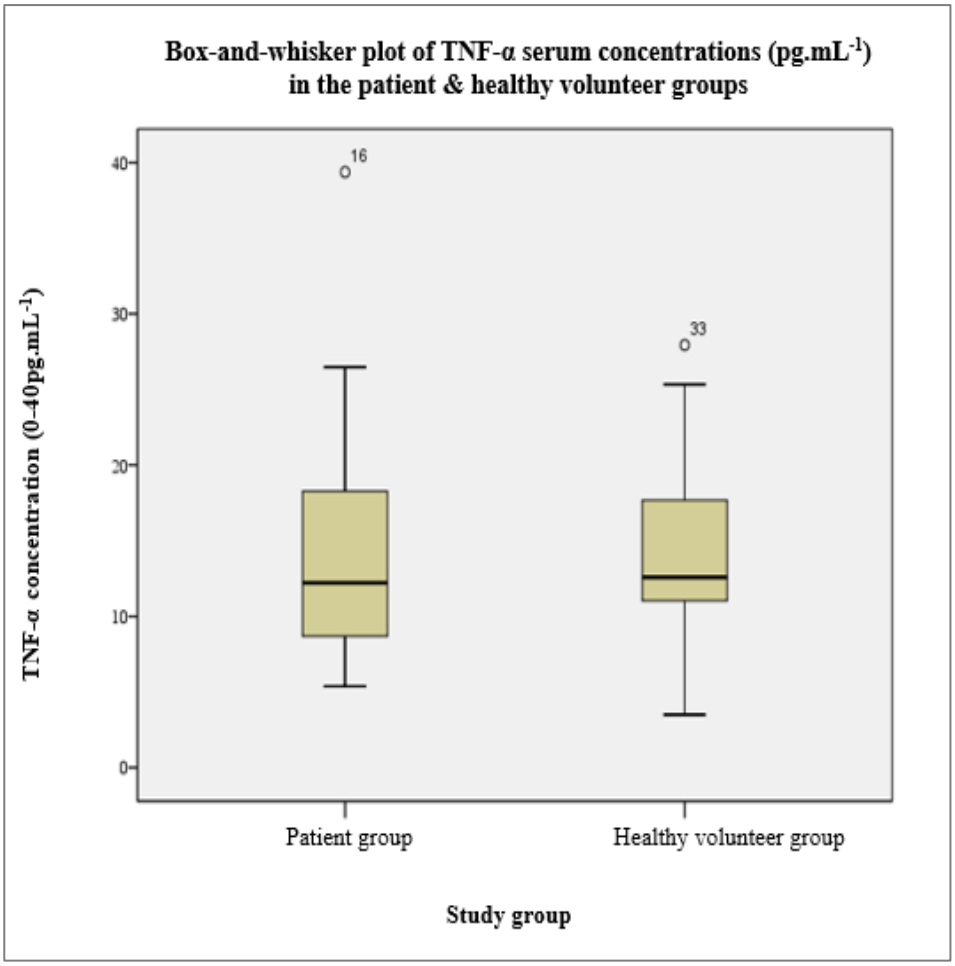
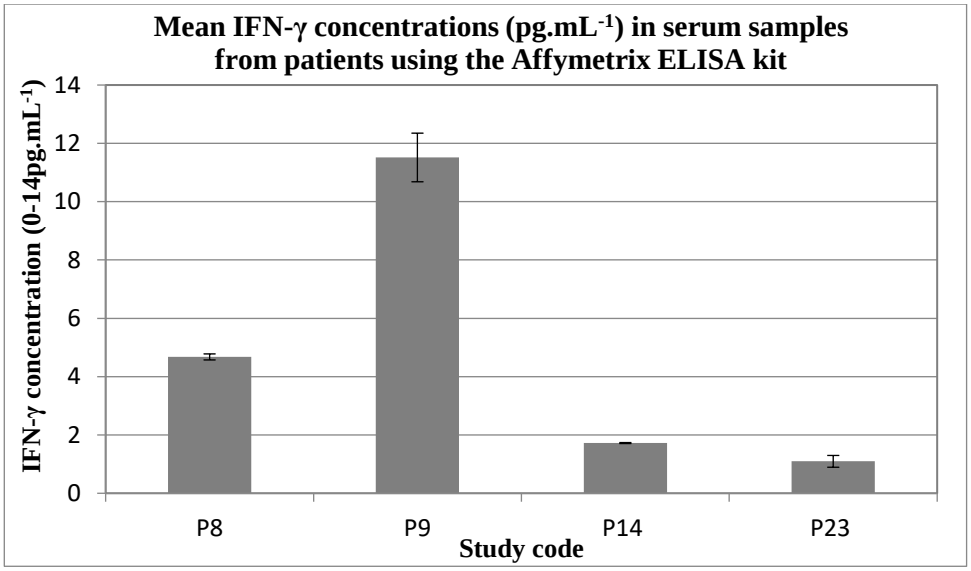


Figure 3.14: Box-and-whisker plot of TNF-α serum concentrations (pg.mL^{-1}) in the patient and healthy volunteer groups (n=38, 20 patients and 18 healthy volunteers). The boxplot gave a visual depiction of the distribution of data in both groups tested with means of serum replicates plotted. Boxes represent the interquartile range (25th to 75th percentile) and the line across the box indicates the median. Outliers were observed in both group (circular dots were outliers. TNF-α, tumour necrosis factor-alpha; n, number of subjects.

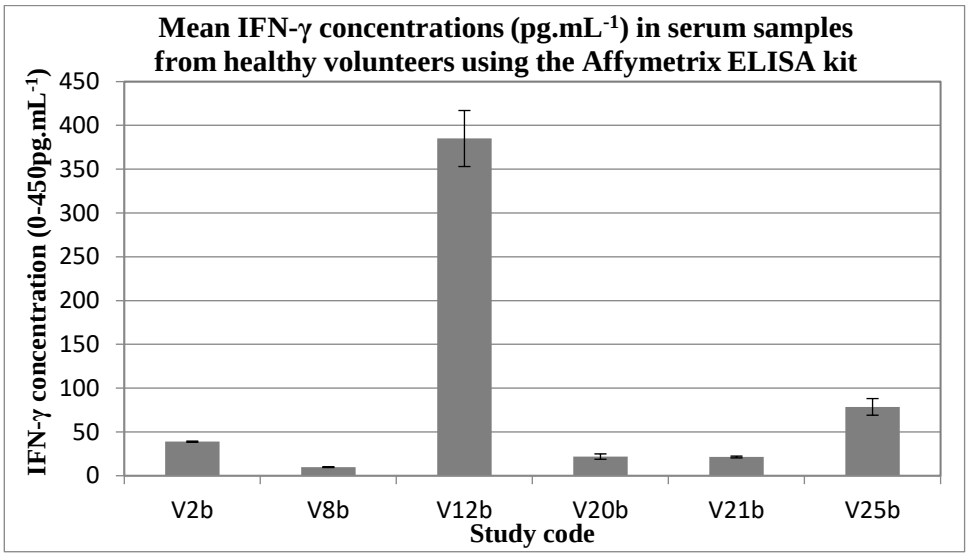
3.5 IFN-γ concentrations in the study groups with statistical analysis

The IFN-γ data was presented using histograms based on the specimen type tested, either serum or SF (Figure 3.15 and Figure 3.16). The Affymetrix eBiosciences human IFN-γ precoated ELISA kit (catalogue #: BMS228) was used for all IFN-γ cytokine quantification. The total number of participants for the analysis of serum IFN-γ in the current study was 38 (20 patients and 18 healthy volunteers) where the mean ± SD concentration determined for the patient group was 0.95 ± 2.72pg.mL^{-1} and 30.86 ± 90.69pg.mL^{-1} for the healthy volunteer group. The patients with detectable concentrations of serum IFN-γ were P8, P9, P14 and P23, while P1 to P7, P10 to P13, P15, P26, P30, P32 and P38 had undetectable concentrations of IFN-γ. The healthy volunteers with detectable concentrations of serum IFN-γ were V2b, V8b, V12b, V20b, V21b and V25b, while V1b, V4b, V11b, V13b, V15b to V19b, V23b, V24b and V26b had undetectable concentrations of IFN-γ. The healthy volunteer with the highest IFN-γ mean ± SD serum concentration was V12b with 385.04 ± 31.99pg.mL^{-1}. There were 18 patients (P1 to P9, P11 to P15, P23, P26, P30 and P32) that participated in SF analysis. Most patients had undetectable concentrations of IFN-γ in SF, except P7, P11, P14 and P23 which had detectable concentrations (Figure 3.16). P10 had a dry knee so no SF was available for analysis. The patient with the highest IFN-γ mean ± SD concentration of SF was P7 with 55.20 ± 0.33pg.mL^{-1}.

For serum IFN-γ, there were outliers present in the data, as assessed by inspection of a boxplot (Figure 3.17). Medians for each group were 0.00pg.mL^{-1} for the patient group and 0.00pg.mL^{-1} for the healthy volunteer group. The IFN-γ concentrations in the patient and healthy volunteer groups were not normally distributed, as assessed by Shapiro-Wilk's test (P<0.05). A Mann-Whitney U test was conducted to determine if there were differences in median IFN-γ concentrations between the patient and the healthy volunteer group. Distributions of IFN-γ concentrations in the patient and healthy volunteer groups were similar, as assessed by visual inspection. Median IFN-γ concentrations for the patient group (0.00pg.mL^{-1}) and the healthy volunteer group (0.00pg.mL^{-1}) was not statistically significant, U= 145, z= -1.321, P= 0.317.



3.15a. Mean IFN-γ concentrations (pg.mL^{-1}) in serum from patients (error bars denote SD).



3.15b. Mean IFN-γ concentrations (pg.mL^{-1}) in serum from healthy volunteers (error bars denote SD).

Figure 3.15a. Mean IFN-γ concentrations (pg.mL^{-1}) in serum from patients; 3.15b. Mean IFN-γ concentrations (pg.mL^{-1}) in serum from healthy volunteers. All samples were tested in duplicate using the Affymetrix eBiosciences human IFN-γ ELISA kit (catalogue #: BMS228). Error bars were calculated using the SD of sample duplicate replicates (n=38, 20 patients and 18 healthy volunteers). ELISA, enzyme linked immunosorbent assay; IFN-γ, Interferon-gamma; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

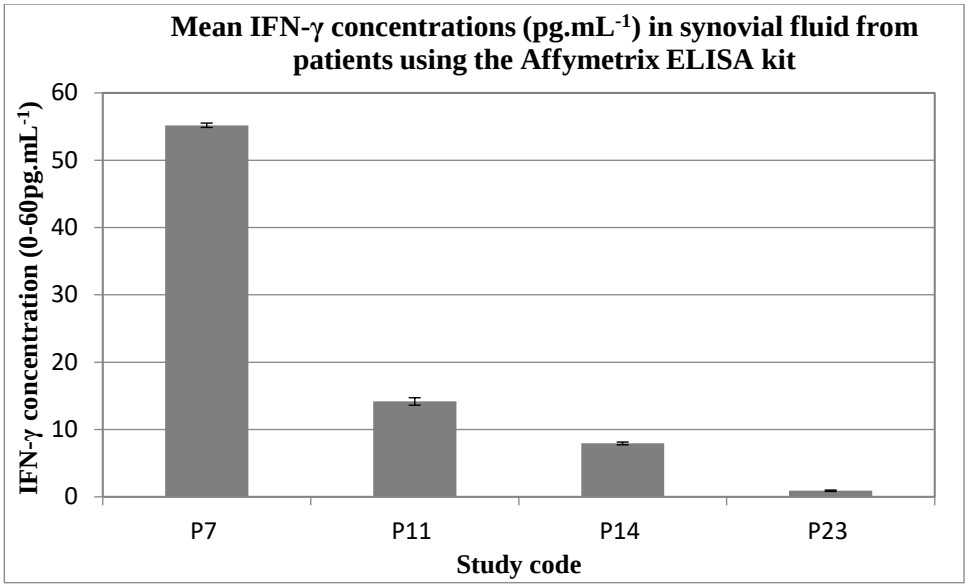


Figure 3.16: Mean IFN-γ concentrations (pg.mL^{-1}) in synovial fluid samples from patients using the Affymetrix eBiosciences human IFN-γ ELISA kit (catalogue #: BMS228) (n=18). All synovial fluid samples were tested in duplicate replicates. Of the 18 patients synovial fluid samples tested, only 4 patients had detectable concentrations of IFN-γ (P7, P11, P14 and P23). Error bars were calculated using the SD of the sample replicates. ELISA, enzyme linked immunosorbent assay; IFN-γ, Interferon-gamma; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

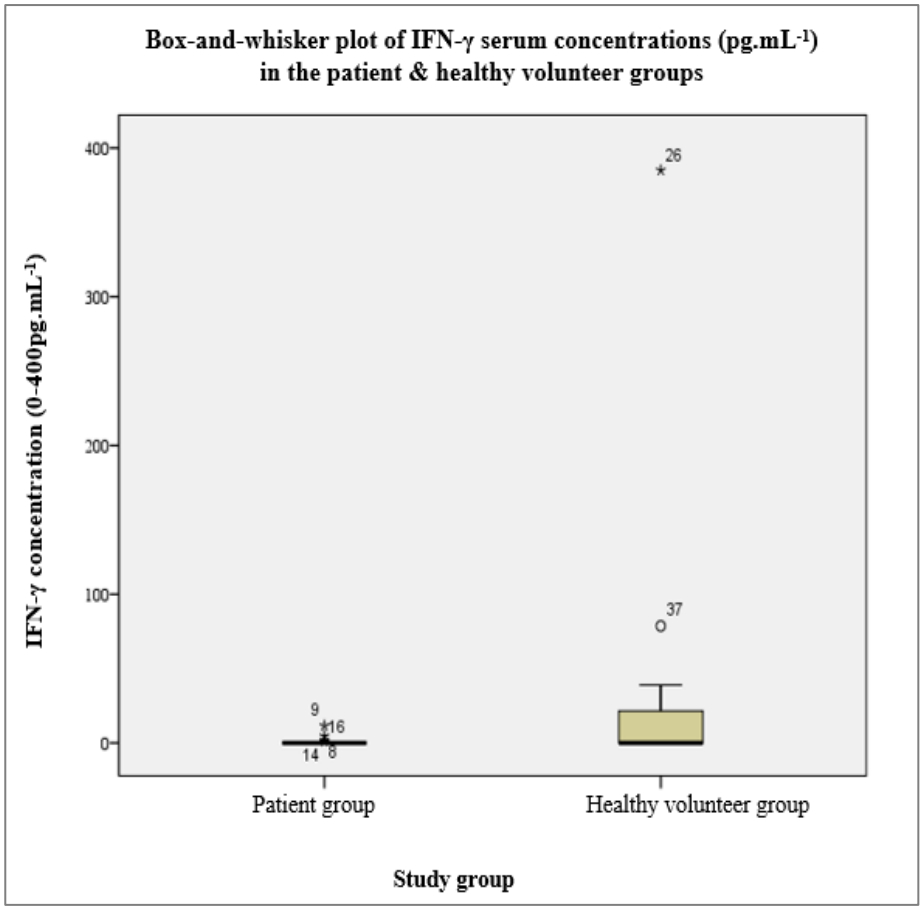


Figure 3.17: Box-and-whisker plot of IFN-γ serum concentrations (pg.mL^{-1}) in the patient and healthy volunteer groups (n=38, 20 patients and 18 healthy volunteers). The boxplot gave a visual depiction of the distribution of data in both groups tested with means of serum replicates plotted. Boxes represent the interquartile range (25th to 75th percentile) and the line across the box indicates the median. Outliers were observed in both groups (circular dots were outliers and an asterix represented an extreme outlier). IFN-γ, Interferon-gamma; n, number of subjects.

3.6 IL-13 concentrations in the study groups with statistical analysis

The IL-13 data was presented using histograms based on the specimen type tested (Figure 3.18 and Figure 3.19). The Affymetrix eBiosciences human IL-13 precoated ELISA kit (catalogue #: BMS231/3) was used for all IL-13 cytokine quantification. The total number of participants for the analysis of serum IL-13 in the current study was 38 (20 patients and 18 healthy volunteers) where the mean ± SD concentration determined for the patient group was 15.83 ± 14.49pg.mL^{-1} and 30.68 ± 99.29pg.mL^{-1} for the healthy volunteer group. The patients with detectable concentrations of serum IL-13 were P4, P7, P11, P15 and P30, while P1 to P3, P5, P6, P8 to P10, P12 to P14, P23, P26, P32 and P38 had undetectable concentrations of IL-13. The healthy volunteers with detectable concentrations of serum IL-13 were V2b, V12b, V16b and V25b, while V1b, V4b, V11b, V13b, V15b, V17b to V21b, V23b, V24b and V26b had undetectable concentrations of IL-13. IL-13 serum mean ± SD concentrations in the patient group ranged from 7.35 ± 0.67pg.mL^{-1} to 185.73 ± 21.14pg.mL^{-1}. IL-13 serum mean ± SD concentrations in the healthy volunteer group ranged from 7.41 ± 0.55pg.mL^{-1} to 410.21 ± 17.94pg.mL^{-1}. There were 18 patients (P1 to P9, P11 to P15, P23, P26, P30 and P32) that participated in SF analysis. Most patients had undetectable concentrations of IL-13 in SF, except P7, P11 and P15 which had detectable concentrations (Figure 3.19). P10 had a dry knee so no SF was available for analysis.

For serum IL-13, there were outliers present in the data, as assessed by inspection of a boxplot (Figure 3.20). Medians for each group were 0.00pg.mL^{-1} for the patient group and 0.00pg.mL^{-1} for the healthy volunteer group. The IL-13 concentrations in the patient and healthy volunteer groups were not normally distributed, as assessed by Shapiro-Wilk's test (P<0.05). A Mann-Whitney U test was conducted to determine if there were differences in median IL-13 concentrations between the patient and the healthy volunteer group. Distributions of IL-13 concentrations in the patient and healthy volunteer groups were similar, as assessed by visual inspection. Median IFN-γ concentrations for the patient group (0.00pg.mL^{-1}) and the healthy volunteer group (0.00pg.mL^{-1}) was not statistically significant, U= 176, z= -0.157, P= 0.919.

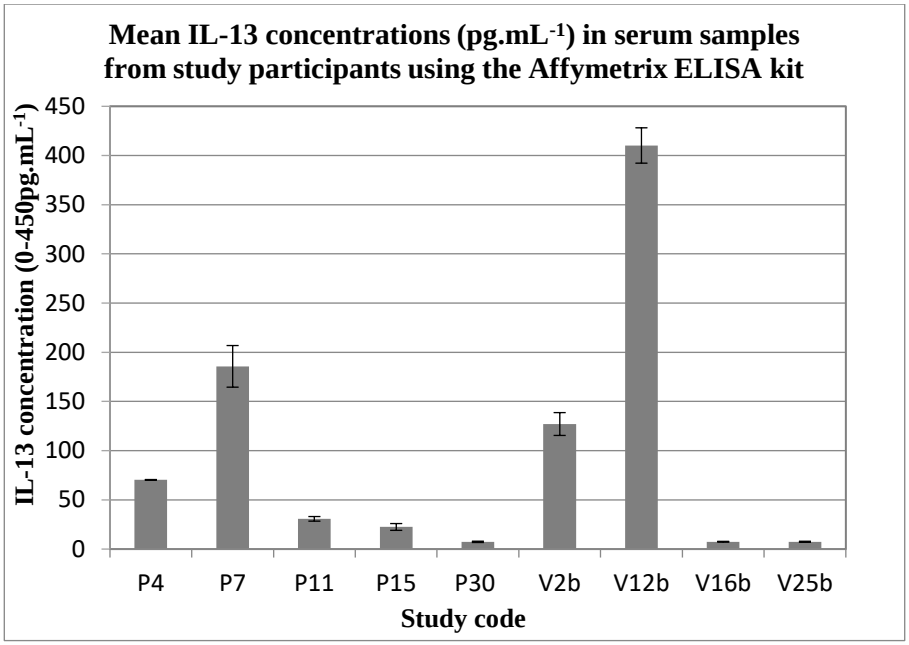


Figure 3.18: Mean IL-13 concentrations (pg.mL^{-1}) in serum samples from study participants using the Affymetrix ELISA kit. All serum samples from patients and healthy volunteers were tested in duplicate using the Affymetrix eBiosciences human IL-13 ELISA kit (catalogue #: BMS231/3). The 5-parameter curve fit was used for data analysis (n=38, 20 patients and 18 healthy volunteers). ELISA, enzyme linked immunosorbent assay; IL-13, interleukin-13; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

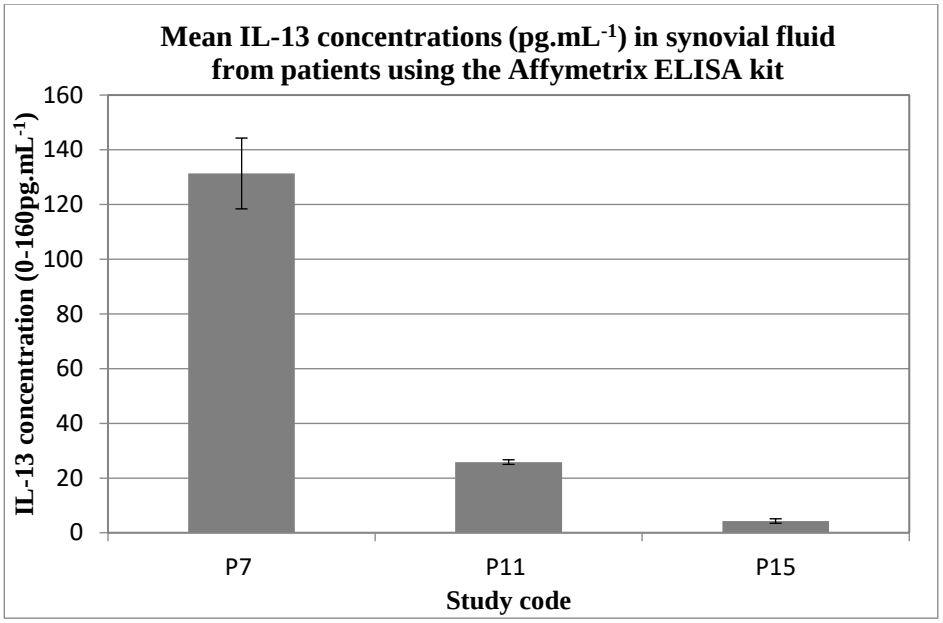


Figure 3.19: Mean IL-13 concentrations (pg.mL^{-1}) in synovial fluid samples from patients using the Affymetrix eBiosciences human IL-13 ELISA kit (catalogue #: BMS231/3) (n=18). All synovial fluid samples were tested in duplicate replicates. Of the 18 patients synovial fluid samples tested, only 3 patients (P7, P11, and P15) had detectable concentrations of IL-13. Error bars were calculated using the SD of the sample replicates. ELISA, enzyme linked immunosorbent assay; IL-13, interleukin-13; pg.mL^{-1}, picograms per millilitre; n, number of subjects; SD, standard deviation.

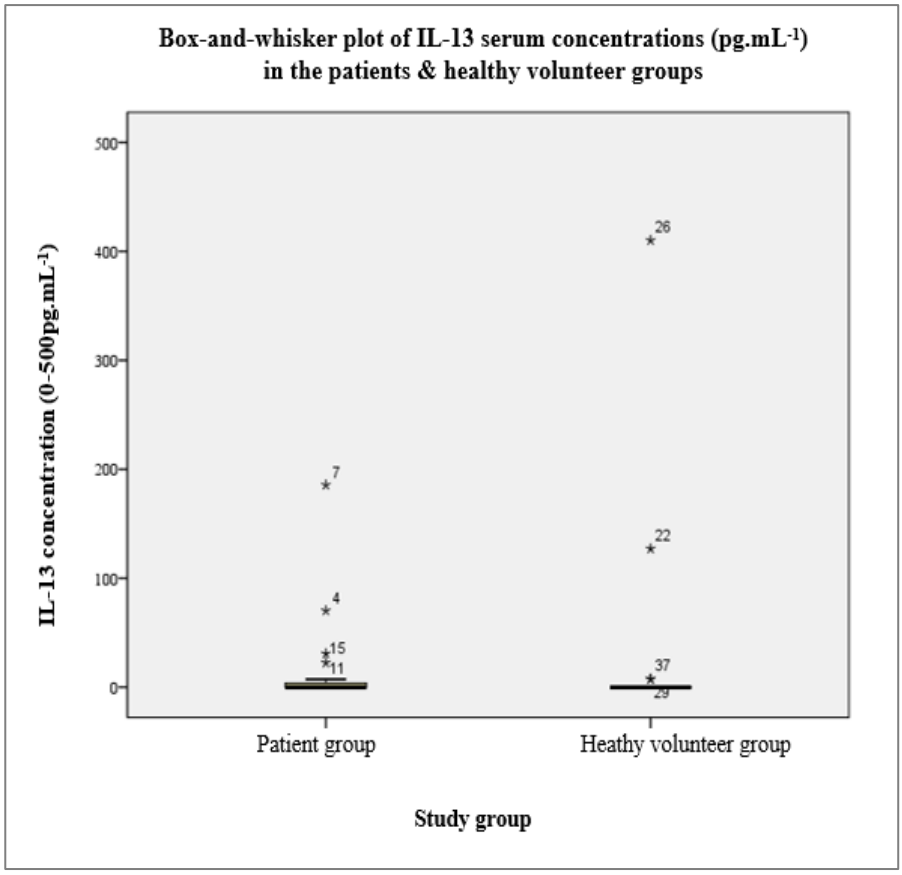


Figure 3.20: Box-and-whisker plot of IL-13 serum concentrations (pg.mL^{-1}) in the patient and healthy volunteer groups (n=38, 20 patients and 18 healthy volunteers). The boxplot gave a visual depiction of the distribution of data in both groups tested with means of serum replicates plotted. Boxes represent the interquartile range (25th to 75th percentile) and the line across the box indicates the median. Outliers were observed in both groups (extreme outliers were indicated with an asterix). IL-13, interleukin-13; n, number of subjects.

3.7 Interesting cytokine profiles in the study

The two patients in the current study (P7 and P11) had the highest concentrations of cytokines in their SF samples (Table 3.1). P7 had cytokine concentrations in SF as follows: IFN-γ was 55.20 ± 0.33pg.mL^{-1}; IL-6 was 2258.39 ± 10.10pg.mL^{-1}; TNF-α was 25.08 ± 0.91pg.mL^{-1}; IL-10 was 227.71 ± 5.94pg.mL^{-1}; IL-13 was 131.35 ± 12.95pg.mL^{-1}; and IL-17A was 9.97 ± 2.75pg.mL^{-1}. These SF cytokine concentrations were higher than matched serum samples, indicating that the SF cytokine concentrations were more reflective of the inflammatory state of KOA for these two patients. In the present study, P7 and P11, may have suffered from an inflammatory form of KOA based on analysis of their cytokine profiles. The pro-inflammatory cytokine, IL-6, was present at the highest concentrations in these two patients, indicating that they may suffer from an inflammatory form of KOA. The healthy volunteers in the current study (V2b, V12b and V25b) had the highest concentrations of cytokines tested in their serum samples (Table 3.1). The cytokines, IFN-γ and IL-13, were present at the highest concentrations in these healthy volunteers possibly indicating early arthritis or autoimmune disease. The healthy volunteer V2b had an IFN-γ and IL-13 concentration of 38.85 ± 0.70pg.mL^{-1} and 127.11 ± 11.60pg.mL^{-1}, respectively. The healthy volunteer V12b had an IFN-γ and IL-13 concentration of 385.04 ± 31.99pg.mL^{-1} and 410.21 ± 17.94pg.mL-1.mL^{-1}, respectively. The healthy volunteer V25b had an IFN-γ and IL-13 concentration of 78.60 ± 9.48pg.mL^{-1} and 7.41 ± 0.55pg.mL^{-}1, respectively.

Table 3.1: Interesting cytokine profiles in patient SF and healthy volunteer serum samples. Of the 18 patients tested, there were 2 patients (P7 and P11) with knee osteoarthritis that had high cytokine concentrations present in their SF samples. In the healthy volunteer group, there were 3 patients (V2b, V12b and V25b) with high concentrations of the cytokines tested in their serum samples. IL, interleukin; IFN-γ, Interferon-gamma; TNF- α, tumor necrosis factor-alpha; SF, synovial fluid; mean ± SD, mean ± standard deviation.

Study code	Cytokine	Cytokine concentration (mean ± SD)	Specimen type
P7	IFN-γ	55.20 ± 0.33pg.mL^{-1}	SF
	IL-6	2258.39 ± 10.10pg.mL^{-1}	SF
	TNF-α	25.08 ± 0.91pg.mL^{-1}	SF
	IL-10	227.71 ± 5.94pg.mL^{-1}	SF
	IL-13	131.35 ± 12.95pg.mL^{-1}	SF
	IL-17A	9.97 ± 2.75pg.mL^{-1}	SF
P11	IFN-γ	14.17 ± 0.55pg.mL^{-1}	SF
	IL-6	2021.82 ± 26.69pg.mL^{-1}	SF
	TNF-α	Below 2.00pg.mL^{-1} (ELISA kit sensitivity)	SF
	IL-10	331.38 ± 2.46pg.mL^{-1}	SF
	IL-13	25.83 ± 0.84pg.mL^{-1}	SF
	IL-17A	2.40 ± 0.03pg.mL^{-1}	SF
V2b	IFN-γ	38.85 ± 0.70pg.mL^{-1}	Serum
	IL-6	2.15 ± 0.16pg.mL^{-1}	Serum
	TNF-α	17.20 ± 0.51pg.mL^{-1}	Serum
	IL-10	35.93 ± 0.65pg.mL^{-1}	Serum
	IL-13	127.11 ± 11.60pg.mL^{-1}	Serum
	IL-17A	15.57 ± 1.32pg.mL^{-1}	Serum
V12b	IFN-γ	385.04 ± 31.99pg.mL^{-1}	Serum
	IL-6	2.49 ± 0.04pg.mL^{-1}	Serum
	TNF-α	11.86 ± 1.62pg.mL^{-1}	Serum
	IL-10	29.05 ± 8.08pg.mL^{-1}	Serum
	IL-13	410.21 ± 17.94pg.mL^{-1}	Serum
	IL-17A	7.39 ± 0.69pg.mL^{-1}	Serum
V25b	IFN-γ	78.60 ± 9.48pg.mL^{-1}	Serum
	IL-6	1.77 ± 0.00pg.mL^{-1}	Serum
	TNF-α	22.56 ± 4.53pg.mL^{-1}	Serum
	IL-10	42.18 ± 7.53pg.mL^{-1}	Serum
	IL-13	7.41 ± 0.55pg.mL^{-1}	Serum

3.8 Cytokine concentrations at different time points in the study

Selected healthy volunteers provided blood samples at two time points to assess the difference in certain cytokines over time. The selection criteria was based on availability of blood samples at the two time points. There was twenty months between the first (November 2014) and second blood collection (July 2015) for the healthy volunteer group. The presence of "a" after subject number indicated the blood sample was from the first collection, while "b" indicated that the blood sample was from the second collection. Each participant in the study was randomly assigned a unique number for identification purposes. There was sufficient serum samples available at both time-points for the analysis of 3 cytokines (IFN-γ, IL-13 and IL-6). For IFN-γ and IL-13, the only serum samples available at both time-points was from the healthy volunteer with the study code, V2. For IFN-γ, V2a had a serum mean ± SD concentration of 27.44 ± 1.37pg.mL^{-1} (duplicate replicates), while V2b had a mean ± SD concentration of 38.85 ± 0.70pg.mL^{-1}. There was a significant difference between the IFN-γ means for V2 at the different time-points where P= 0.015 (P<0.05). For IL-13, V2a had a serum mean ± SD concentration of 169.89 ± 13.37pg.mL^{-1} (duplicate replicates), while V2b had a mean ± SD concentration of 127.11 ± 11.60pg.mL^{-1}. There was no significant difference between the means for V2 at the different time-points where P= 0.999 (P>0.05).

A paired-samples *t*-test was conducted to compare IL-6 mean serum concentrations in healthy volunteers at the 2 time-points, "a" and "b". The IL-6 serum mean concentrations were higher at time-point "a" (mean ± SD of 1.97 ± 0.35pg.mL^{-1}) than time-point "b" (mean ± SD of 1.48 ± 0.44pg.mL^{-1}); a significant difference of 0.50pg.mL^{-1} (95% CI, 0.16 to 0.84), t (8) = 3.40, P<0.05. There were 4 healthy volunteers where there were high variations in IL-6 concentrations from the 2 time-points (V4 with a mean ± SD concentration of 1.79 ± 0.05pg.mL^{-1} at time-point "a" and 1.14 ± 0.05pg.mL^{-1} at time-point "b"; V11 with 2.50 ± 0.05pg.mL^{-1} at time-point "a" and 1.86 ± 0.09pg.mL^{-1} at time-point "b"; V15 with 2.04 ± 0.12pg.mL^{-1} at time-point "a" and 0.97 ± 0.05pg.mL^{-1} at time-point "b"; and V19 with 2.05 ± 0.29pg.mL^{-1} at time-point "a" and 0.78 ± 0.07pg.mL^{-1} at time-point "b" (Figure 3.21).

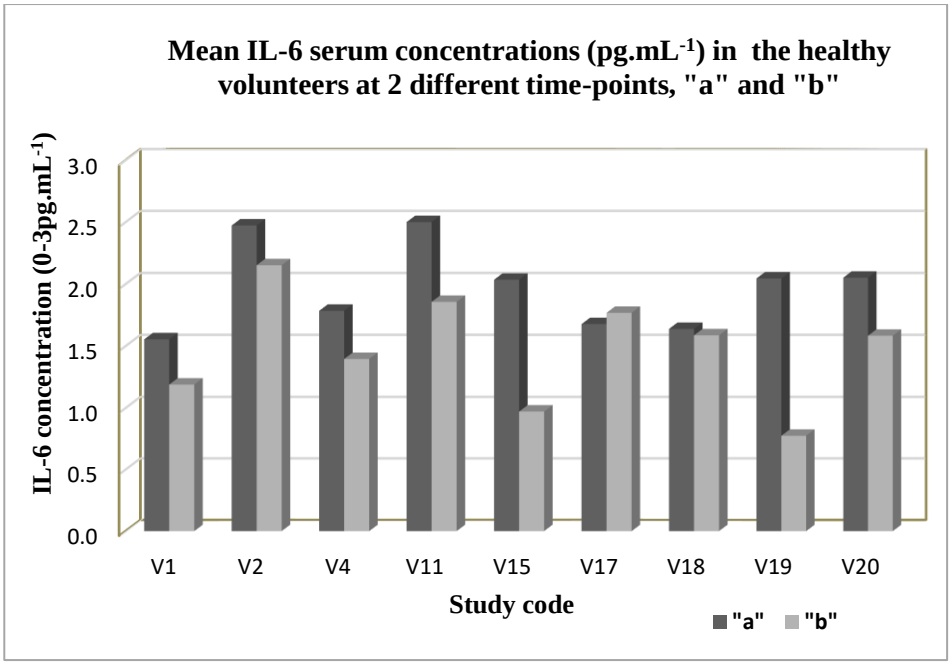


Figure 3.21: Mean serum IL-6 concentrations (pg.mL^{-1}) in healthy volunteers at 2 different time-points, "a" and "b". There was a 20 month period between collection "a" and "b". For the volunteer group, "a" indicated that the serum sample was from the first collection and "b" indicated that the serum sample was from the second collection. There were 9 healthy volunteers that participated in this experiment, n=9. IL-6, interleukin-6; V, Volunteer.

3.9 Comparison of IL-6 concentrations in matched plasma and serum samples

The IL-6 concentrations in matched plasma and serum samples from study participants (*n*=12, 6 patients and 6 healthy volunteers) were compared to determine the best specimen type for cytokine analysis. The serum and plasma samples were tested in triplicate using the R&D Systems precoated IL-6 ELISA kit (catalogue #: D6050) (Table 3.2). IL-6 concentrations were similar in matched plasma and serum samples. Therefore, serum was selected for cytokine analysis in the study.

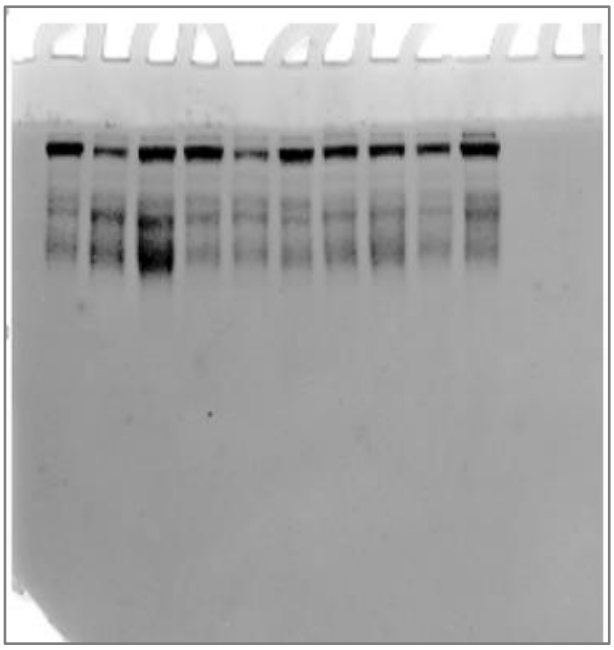
Table 3.2: IL-6 mean ± SD concentrations (pg.mL^{-1}) in matched plasma and serum samples from KOA patients and healthy volunteers. IL-6, interleukin-6; mean ± SD, mean ± standard deviation; KOA, knee osteoarthritis.

Study code	Specimen type	IL-6 concentration (mean ± SD)
P1	Plasma	1.50 ± 0.09pg.mL^{-1}
	Serum	1.74 ± 0.21pg.mL^{-1}
P2	Plasma	9.25 ± 0.42pg.mL^{-1}
	Serum	11.09 ± 0.36pg.mL^{-1}
P3	Plasma	5.28 ± 0.24pg.mL^{-1}
	Serum	6.11 ± 0.37pg.mL^{-1}
P4	Plasma	2.18 ± 0.22pg.mL^{-1}
	Serum	2.09 ± 0.20pg.mL^{-1}
P5	Plasma	3.06 ± 0.22pg.mL^{-1}
	Serum	3.33 ± 0.25pg.mL^{-1}
P7	Plasma	9.11 ± 0.80pg.mL^{-1}
	Serum	9.80 ± 0.50pg.mL^{-1}
V1b	Plasma	1.11 ± 0.10pg.mL^{-1}
	Serum	1.19 ± 0.07pg.mL^{-1}
V4b	Plasma	1.20 ± 0.06pg.mL^{-1}
	Serum	1.14 ± 0.05pg.mL^{-1}
V11b	Plasma	1.70 ± 0.16pg.mL^{-1}
	Serum	1.86 ± 0.09pg.mL^{-1}
V15b	Plasma	1.00 ± 0.16pg.mL^{-1}
	Serum	0.97 ± 0.05pg.mL^{-1}
V19b	Plasma	0.91 ± 0.24pg.mL^{-1}
	Serum	0.78 ± 0.07pg.mL^{-1}
V23b	Plasma	1.11 ± 0.21pg.mL^{-1}
	Serum	1.11 ± 0.08pg.mL^{-1}

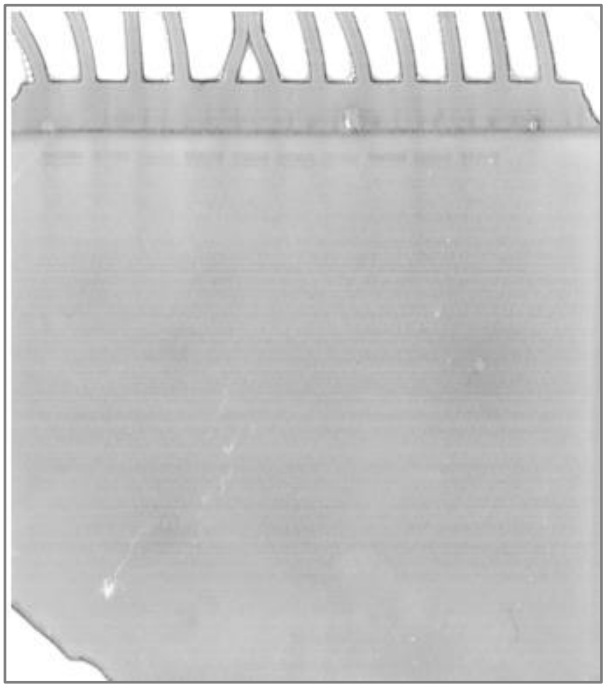
3.10 Gelatin zymography results

There were two negative control gels included in each test: the first gel was incubated in EDTA incubation buffer; and the second gel had no $CaCl_2$ incorporated in resolving gelatin zymogram recipe. The negative control gelatin zymograms inhibited MMPs as no bands of degradation were observed (Figure 3.22a and Figure 3.22b). The MMPs could not function without calcium incorporated in the incubation buffer and soaking the gels in EDTA incubation buffer inhibited the MMPs. This verified that bands of degradation were definitely MMPs based on the properties of EDTA inhibiting MMPs and that MMPs require calcium to function. Gelatin zymogram images that were suitable for Image J analysis had high quality with low background noise (Figure 3.23 and Figure 3.24). AlphaView image analysis was used to determine band intensity values. Images were displayed in square pixels which gave a representation of the total MMP-9 concentration in plasma samples from patients and healthy volunteers (the greater the intensity of the band, the greater the concentration of total MMP-9 in the plasma samples).

The 92kDa and 82kDa bands were not separated adequately using gelatin zymography so the combination of these bands represented total MMP-9. Active MMP-2 (64kDa) was detected in patient and healthy volunteer plasma samples. The images suitable for Image J analysis had high variability between the duplicate replicates of the plasma samples tested with high standard deviations reported (Table 3.3). The patient, P18, had the highest mean ± SD band intensity value of 7036.07 ± 2071.36 square pixels in the plasma samples tested. This suggested that P18 had high concentrations of total MMP-9 which may be somewhat responsible for the damage experienced in their knee joint. Gelatin zymogram images that were unsuitable for Image J analysis due to poor quality acted as qualitative data to illustrate the presence of the 125kDa band (Figure 3.25a) and to visually observe the greater intensity of bands for total MMP-9 in patient plasma samples (Figure 3.25a and Figure 3.25b).

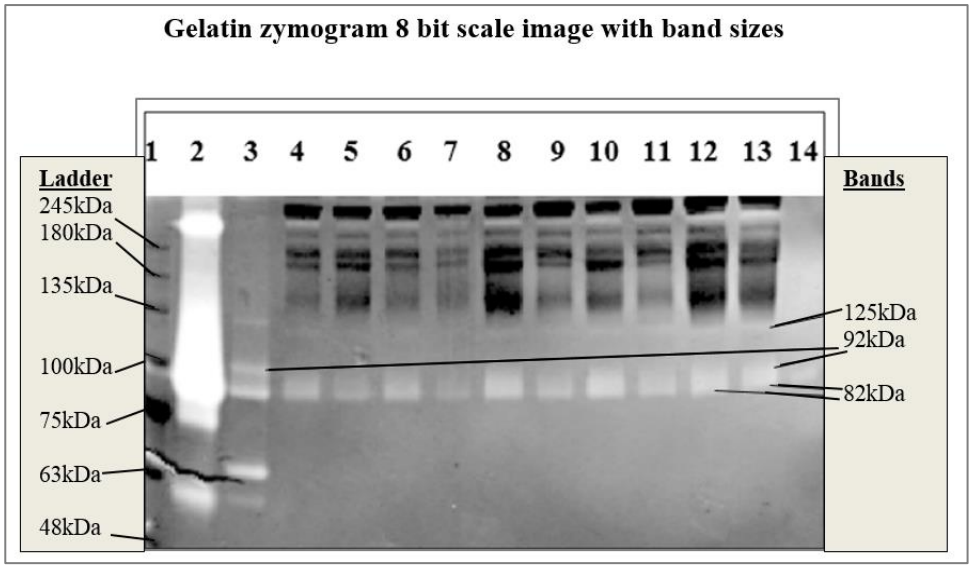


3.22a. EDTA negative control gelatin zymogram with no bands detected.

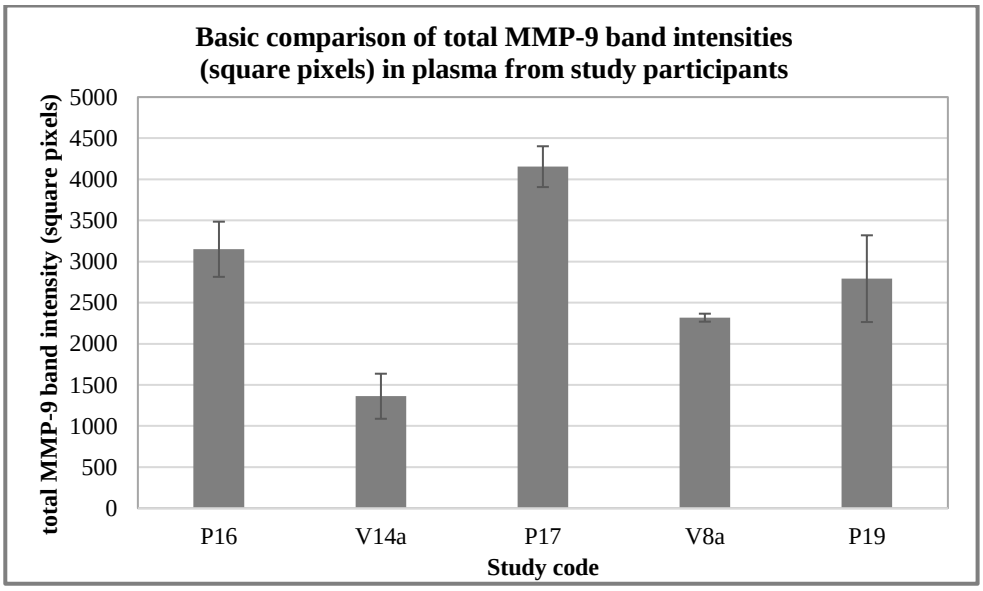


3.22b. $CaCl_2$ negative control gelatin zymogram with no bands detected.

Figure 3.22a: EDTA negative control gelatin zymogram with no bands detected; 3.22b: $CaCl_2$ negative control gelatin zymogram with no bands detected. The figure illustrates images of negative control results used in gelatin zymography analysis. EDTA, ethylenediaminetetraacetic acid; $CaCl_2$, calcium chloride.

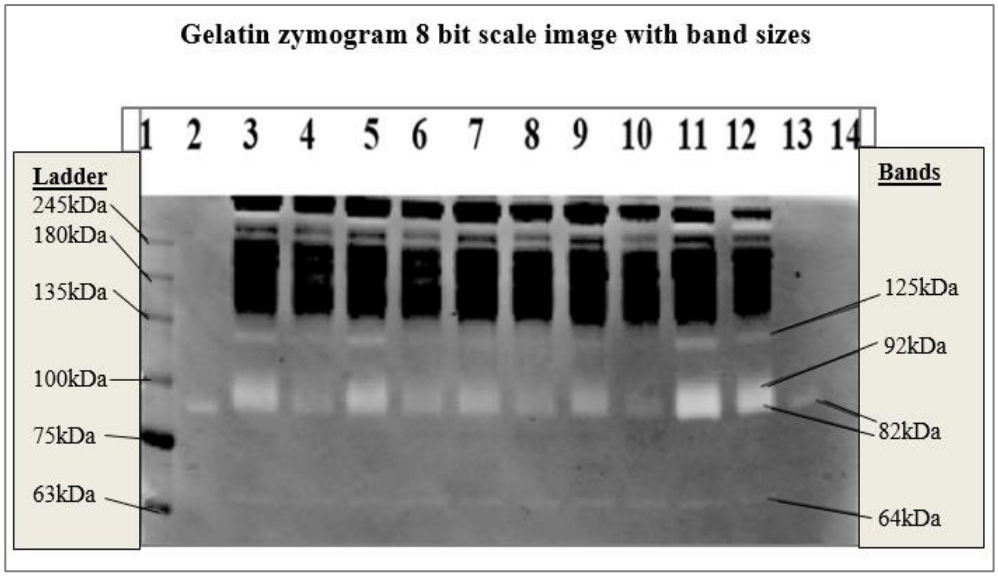


3.23a. Gelatin zymogram 8 bit grey scale image with band sizes.

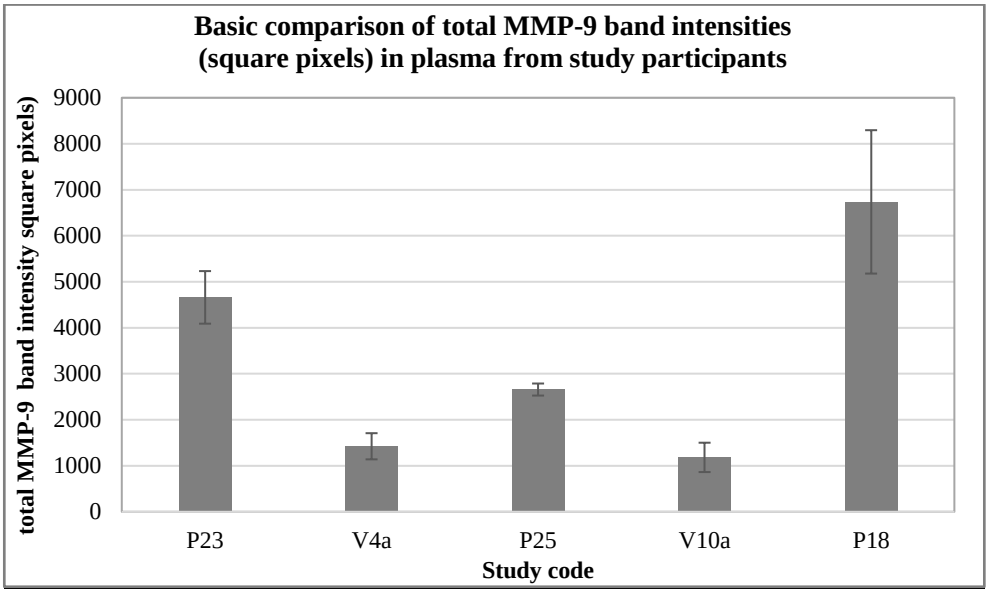


3.23b. Basic comparison of total MMP-9 band intensities (square pixels) in plasma from study participants.

Figure 3.23: a. Gelatin zymogram 8 bit grey scale image with band sizes; 3.23b Basic comparison of total MMP-9 band intensities (square pixels) in plasma (duplicate replicates) from study participants (n=5, 3 patients and 2 healthy volunteers). For 3.23a., lanes 1 to 14 had the following added: 1, Applichem 10-245kDa ladder; 2, WB MMP-9; 3, WB MMP-2; 4, P16; 5, V14a; 6, P16; 7, V14a; 8, P17; 9, V8a; 10, P17; 11, V8a; 12, P19; 13, P19; 14, MMP standard. L, left; R, right; MMP, matrix metalloproteinase; WB MMP-2 & -9, Bio-Techne MMP-2 and -9 standards; KOA, knee osteoarthritis; n, number of study participants.



3.24a. Gelatin zymogram 8 bit grey scale image with band sizes.

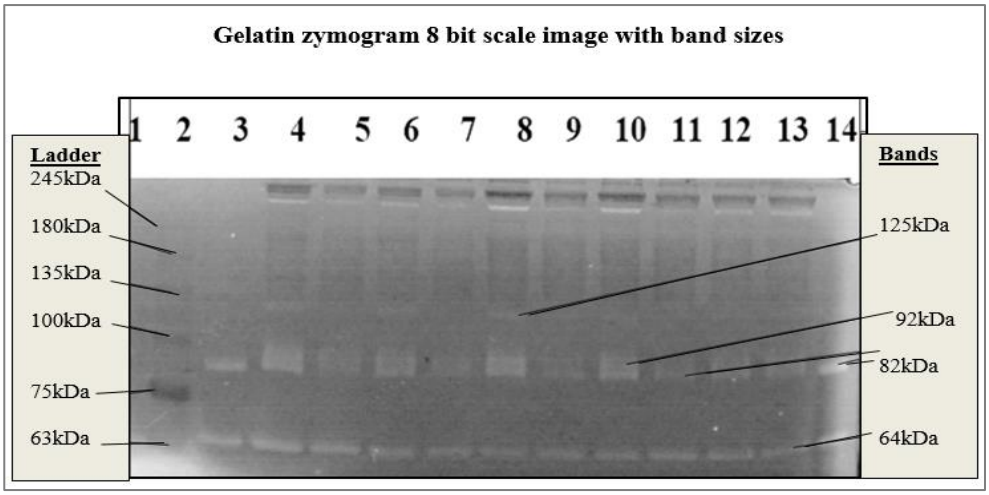


3.24b. Basic comparison of total MMP-9 band intensities (square pixels) in plasma from study participants.

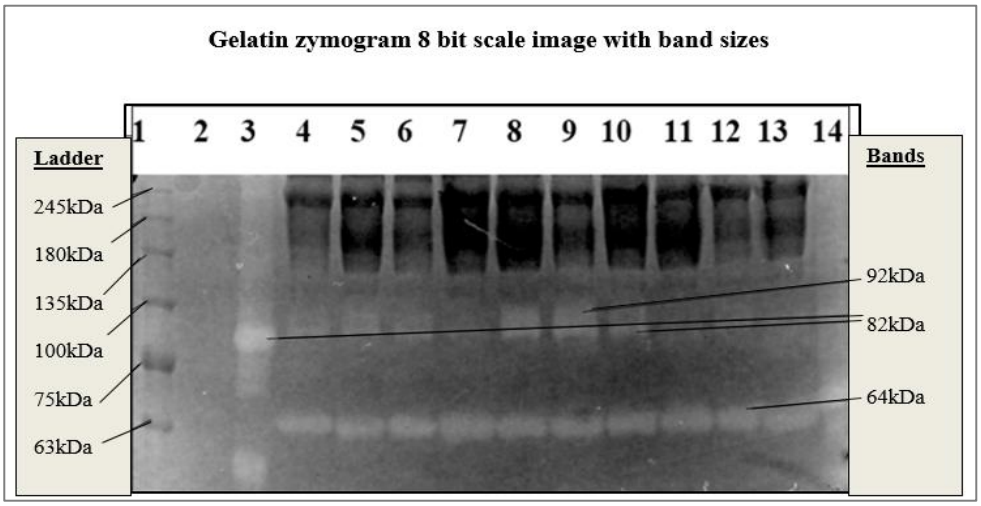
Figure 3.24: a. Gelatin zymogram 8 bit grey scale image with band sizes; 3.24b Basic comparison of total MMP-9 band intensities (square pixels) in plasma (duplicate replicates) from study participants (n=5, 3 patients and 2 healthy volunteers). For 3.24a., lanes 1 to 14 had the following added: Applichem 10 to 245kDa ladder; 2, MMP standard; 3, P23; 4, V4a; 5, P23; 6, V4a; 7, P25; 8, V10a; 9, P25; 10, V10a; 11, P18; 12, P18; 13, MMP standard; 14, none. L, left; R, right; MMP, matrix metalloproteinase; KOA, knee osteoarthritis; n, number of participants.

Table 3.3: Plasma total MMP-9 mean ± SD band intensities (square pixels) in study participants. Images were displayed in square pixels which gave a representation of the total MMP-9 concentration in plasma samples. There was a significant difference in total MMP-9 means between the patient and healthy volunteer groups ($P<0.05$). The mean ± SD was 1482.75 ± 478.86 square pixels (n=4) for the patient group and 3887.00 ± 1705.44 square pixels (n=6) for the patient group. MMP-9, matrix metalloproteinase-9; mean ± SD, mean ± standard deviation.

Study Code	**Band intensity of total MMP-9 (Mean ± SD)**
V4a	1265.69 ± 179.15 square pixels
V8a	2183.18 ± 22.18 square pixels
V10a	1111.89 ± 226.86 square pixels
V14a	1370.22 ± 473.99 square pixels
P16	3147.00 ± 277.72 square pixels
P17	3943.79 ± 264.80 square pixels
P18	7036.07 ± 2071.36 square pixels
P19	2369.81 ± 654.88 square pixels
P23	4226.06 ± 388.46 square pixels
P25	2599.26 ± 108.31 square pixels



3.25a. Gelatin zymogram image 1 with band sizes labelled.



3.25b. Gelatin zymogram image 2 with band sizes labelled.

Figure 3.25: a. Gelatin zymogram image 1 with band sizes labelled (n= 5, 3 patients and 2 healthy volunteers); b. Gelatin zymogram image 2 with band sizes labelled (n=10, 5 patients and 5 volunteers). For 3.25a, all plasma were tested in duplicate where lanes 1 to 14 contained: 1, Nothing; 2, Applichem 10-245kDa ladder; 3, MMP standard; 4, P20; 5, V12a; 6, P20; 7, V12a; 8, P21; 9, V2a; 10, P21; 11, V2a; 12, P36; 13, P36; 14, MMP standard. For 3.25b, all plasma was tested singly (no replicates of samples) where lanes 1 to 14 contained: 1, Applichem 10-245kDa ladder; 2, WB MMP-9; 3, WB MMP-2; 4, P22; 5, V15a; 6, P36; 7, V11a; 8, P37; 9, V5a; 10, P40; 11, V9a; 12, P39; 13, V6a; 14, WB MMP-2. MMP standard, matrix metalloproteinase standard; KOA, knee osteoarthritis; n, number of participants; WB MMP-2 & -9, Bio-Techne MMP-2 and -9 standards.

Chapter 4: Discussion

4.1 Summary of key findings

The main findings in the research project were summarised below.

- Both serum IL-6 and serum IL-10 had significant difference ($P<0.05$) between the patient and healthy volunteer groups so these cytokines may be useful as biomarkers for KOA. There was not a significant difference in serum IL-17A, TNF-α, IFN-γ and IL-13 concentrations between the patient and healthy volunteer groups ($P>0.05$). The data suggested that serum IL-17A, TNF-α, IFN-γ and IL-13 may not be suitable to be used as biomarkers for KOA. The analysis of IL-17A, TNF-α, IFN-γ and IL-13, IL-6 and IL-10 in SF samples provided a clearer depiction of the inflammatory state of KOA in the knee joint of patients.
- The study may have indicated that high total MMP-9 concentrations were linked to KOA, although further investigation was required. Although, many of the gelatin zymograms produced were not suitable for ImageJ analysis, visually the 125kDa band was observed the patient samples. The presence of the 125kDa band in plasma using gelatin zymography may be useful for the diagnosis of KOA.
- The potential reasons for differences in individual patient cytokine profiles are complex and interdependent. These include: the inflammatory status of disease encountered by the individual patient may have been classified as inflammatory where there were high concentrations of pro-inflammatory cytokines present (IL-6, TNF-α, IL-17A and IFN-γ); if the patient was on medication at the time of the study this may have affected their cytokine profile, for example lower concentrations of IL-6 would be expected in patients taking the drug, Tocilizumab, which targets IL-6; and whether the cytokine analysed is affected by circadian rhythms may have impacted on cytokine concentrations in blood or synovial fluid samples, where samples were taken from patients and healthy volunteers at different times throughout the day of sample collection.

4.2 Analysis of IL-6 in the study

The current study reported mean ± SD IL-6 serum concentrations of 4.78 ± 4.63pg.mL^{-1} in the patient group (*n*=24) and 1.66 ± 0.64pg.mL^{-1} in the healthy volunteer group (*n*=18). For P7 and P11, serum IL-6 mean ± concentrations were high with 9.80 ± 0.50pg.mL^{-1} recorded for P7 (Figure 3.1) and 22.64 ± 0.45pg.mL^{-1} for P11 (Figure 3.2a). There was a significant difference in IL-6 serum concentrations between the patient and healthy volunteer groups (P<0.05). The data suggested that serum IL-6 may be useful as a biomarker for KOA. IL-6 concentrations less than 2pg.mL^{-1} were classified as "low", while "high" concentrations were greater than 4pg.mL^{-1} (Stannus *et al.,* 2010). The IL-6 serum concentrations reported in the current study were similar to data recorded by Imamura *et al*. (2015) where the mean ± SD concentrations of serum IL-6 was 4.38 ± 4.61pg.mL^{-1} in the KOA patient group (*n*=53) and 2.55 ± 1.06pg.mL^{-1} in the healthy volunteer group (*n*=48) (Imamura *et al*., 2015).

In the current study, 18 patients participated in IL-6 analysis in SF where the mean ± SD concentration was 417.91 ± 658.21pg.mL^{-1}. The highest concentrations of IL-6 reported were in the participants with the study codes, P7 and P11. Both P7 and P11 had the highest SF mean ± SD concentrations of IL-6 with 2258.39 ± 10.10pg.mL^{-1} recorded for P7 and 2021.82 ± 26.69pg.mL^{-1} for P11 (Figure 3.3). The analysis of SF provided a clearer depiction of the inflammatory state of the knee joint in KOA patients. This was evident in P7 and P11 where the pro-inflammatory cytokine, IL-6, played a key role in the inflammatory response. The IL-6 SF concentration determined in the healthy volunteer study group by Tsuchida *et al*. (2014) provided a reference for comparison in the current study (Table 1.1). Tsuchida *et al.* (2014) recorded similar SF results for SF in KOA patients, with IL-6 mean ± SD concentrations in SF of 396.00 ± 508.00pg.mL^{-1} in the patient group (*n*=27) and 64.00 ± 120.00pg.mL^{-1} in healthy SF of the healthy volunteer group (*n*=20). The healthy SF was obtained from donors within 24 hours of post-mortem (Tsuchida *et al.*, 2014).

4.3 Analysis of IL-10 in the study

The IL-10 serum mean ± SD concentrations reported in the current study were 39.165 ± 16.086pg.mL^{-1} in the patient group (*n*=15) and 30.80 ± 5.66pg.mL^{-1} in the healthy volunteer group (*n*=17). Of the 15 KOA patient serum samples analysed for IL-10, only one patient had less than 30.00pg.mL^{-1} of IL-10. The inclusion of a healthy volunteer group in the current study determined the normal serum IL-10 concentrations. According to the mean of the serum data in the healthy volunteer group in the current study, IL-10 concentrations below 30.00pg.mL^{-1} was classified as "low", while "high" concentrations were greater than 30.00pg.mL^{-1}. The normal concentrations of IL-10 in healthy volunteer groups has varied from study to study. For instance, in the study by Imamura *et al*. (2015) the normal mean ± SD concentration reported in the healthy group (*n*=48) was 0.89 ± 0.87pg.mL^{-1} (Table 1.1), while a study by El bassat *et al.* (2013) reported the mean ± SD concentration of 46.90 ± 16.20pg.mL^{-1} for their healthy group (*n*=15).

The IL-10 concentrations were high in many of the patients tested. The range of mean ± SD concentrations of serum IL-10 in the patient group was 26.75 ± 0.57pg.mL^{-1} (P12) to 93.24 ± 0.58pg.mL^{-1} (P11) (Figure 3.5a) and 24.07 ± 0.89pg.mL^{-1} (V19b) to 42.18 ± 7.53pg.mL^{-1} (V25b) in the healthy volunteer group (Figure 3.5b). The patient, P11, had the highest serum IL-10 concentration which suggested that this cytokine was important in the cytokine cascade in this KOA patient. Other patients that reported high mean ± SD concentrations of serum IL-10 were P7 with 31.09 ± 0.51pg.mL^{-1} and P10 with 42.43 ± 4.14pg.mL^{-1}. There was a significant difference in IL-10 serum concentrations between the patient and healthy volunteer groups (P<0.05). The data suggested that serum IL-10 may be useful as a biomarker for KOA. There were 3 KOA patients (*n*=18) that had detectable concentrations of IL-10 in SF (Figure 3.6). The highest mean ± SD concentrations of IL-10 in SF samples were 331.38 ± 2.46pg.mL^{-1} in P11 and 227.71 ± 5.94pg.mL^{-1} in P7 (Figure 3.6). The matched serum and SF samples had high concentrations of IL-10 for P7 and P11.

4.4 Analysis of IL-17A in the study

According to the serum data in the healthy volunteer group in the current study, IL-17A concentrations below 10.00pg.mL^{-1} was classified as "low", while "high" concentrations were greater than 10.00pg.mL^{-1}. Agache *et al*. (2010) reported the IL-17A mean ± SD concentration of 8.73 ± 11.63pg.mL^{-1} for the healthy volunteer group (n=11) which illustrated variations in IL-17A concentrations in healthy individuals. The IL-17A serum mean ± SD concentrations reported in the current study were 8.33 ± 3.81pg.mL^{-1} for the patient group (n=20) and 9.16 ± 5.33pg.mL^{-1} for the healthy volunteer group (n=18). The range of mean ± SD concentrations of serum IL-17A in the patient group was 3.32 ± 0.26pg.mL^{-1} (P7) to 11.61 ± 2.30pg.mL^{-1} (P6) (Figure 3.9a) and 3.37 ± 0.05pg.mL^{-1} (V13b) to 17.15 ± 2.06pg.mL^{-1} (V8b) in the healthy volunteer group (Figure 3.9b). Other patients where high mean ± SD concentrations of IL-17A were reported included P2 with 10.42 ± 0.86pg.mL^{-1}, P4 with 11.82 ± 1.90pg.mL^{-1}, P5 with 10.96 ± 1.27pg.mL^{-1} and P10 with 11.39 ± 1.09pg.mL^{-1} (Figure 3.9a). There was not a significant difference in IL-17A serum concentrations between the patient and healthy volunteer groups (P>0.05). The data suggested that serum IL-17A may not be a suitable biomarker for KOA.

Agache *et al.* (2010) reported similar IL-17A concentrations in their healthy volunteer group (11 healthy volunteers) to data presented for healthy volunteers in the current study. The IL-17A mean ± SD concentration in their healthy control group was 8.73 ± 11.63pg.mL^{-1} (2 healthy volunteers were below the limit of detection of the assay) (Agache *et al.*, 2010). Agache *et al*. (2010) linked high IL-17A concentrations to mild, moderate and severe asthma (85 asthmatic patients participated), mean ± SD concentrations were 14.21 ± 3.62pg.mL^{-1} in mild asthma, 12.22 ± 3.71pg.mL^{-1} in moderate asthma, and 24.72 ± 4.87pg.mL^{-1} in severe asthma (Agache *et al.*, 2010). High IL-17A serum concentrations in healthy volunteers in the current study may indicate they suffer from asthma or another autoimmune disease. The inflammation reported in this study may have been due to inflammation from another source such as gum disease or periodontitis. IL-17 has been associated with RA, periodontal disease, asthma, multiple sclerosis, inflammatory bowel disease, and psoriasis (Yao *et al.*, 2015).

In the current study, the range of detectable SF mean ± SD concentrations of IL-17A in the patient group (*n*=18) was 2.34 ± 0.60pg.mL^{-1} (P8) to 24.16 ± 12.83pg.mL^{-1} (P14) (Figure 3.10). Other patients that reported high IL-17A SF mean ± SD concentrations were P4 with 18.90 ± 0.09pg.mL^{-1}, P5 with 24.06 ± 2.86pg.mL^{-1}, P7 with 9.96 ± 2.75pg.mL^{-1}, P12 with 15.51 ± 0.84pg.mL^{-1}, and P13 with 22.53 ± 0.48pg.mL^{-1} (Figure 3.10). These patients may have suffered from a form of KOA whereby IL-17A was involved in the inflammatory cytokine cascade, although the inflammation may be the result of another source such as gum disease or periodontitis. The normal range for IL-17A in healthy SF was not clearly defined in any journal article reviewed. However, Rosu *et al*. (2012) investigated concentrations of IL-17A in SF samples from a group of patients with early RA (*n*=29) and a control group of patients with KOA (*n*=29). The IL-17A mean ± SD concentration reported for the RA group was 18.77 ± 12.19pg.mL^{-1} and 4.17 ± 1.20pg.mL^{-1} for the control KOA group (Rosu *et al*., 2012). Based on data for IL-17A for SF in KOA patients in the current study and in the study by Rosu *et al*. (2012), greater than 8pg.mL^{-1} was considered to be “high” IL-17A concentrations in SF samples.

4.5 Analysis of TNF-α in the study

The TNF-α serum mean ± SD concentrations reported in the current study were 14.68 ± 8.66pg.mL^{-1} for the patient group (*n*=20) and 14.29 ± 6.58pg.mL^{-1} for the healthy volunteer group (*n*=18). Based on data in the current study, TNF-α concentrations below 15.00pg.mL^{-1} were considered "low", whereas concentrations above 15.00pg.mL^{-1} were considered "high". The highest TNF-α mean ± SD concentration was observed in P23 with 39.38 ± 0.81pg.mL^{-1} (Figure 3.13a). Other patients with high mean ± SD concentrations of TNF-α included P15 with 24.17 ± 1.99pg.mL^{-1}, P26 with 25.49 ± 6.07pg.mL^{-1} and P30 with 26.48 ± 5.36pg.mL^{-1} (Figure 3.13a). In the healthy volunteer group, the highest TNF-α mean ± SD concentration was observed in V20b with 27.94 ± 2.18pg.mL^{-1} (Figure 3.13b). These patients may have had a form of KOA whereby TNF-α was involved in the inflammatory cytokine cascade. The normal range for TNF-α in serum of healthy volunteer groups has varied from study to study. Imamura *et al.* (2015) reported the mean ± SD concentration for TNF-α of 1.40 ± 1.00pg.mL^{-1} for their healthy volunteer group (*n*=48) (Table 1.1) whereas Ebrahimi *et al*. (2009) reported a higher TNF-α mean ± SD concentration in their healthy volunteer group (*n*=13) of 24.27 ± 8.28pg.mL^{-1}.

There was not a significant difference in TNF-α serum concentrations between the patient and healthy volunteer groups (*P*>0.05). The data suggested that serum TNF-α may not be a suitable biomarker for KOA. The TNF-α SF concentration determined in the healthy volunteer study group by Tsuchida *et al*. (2014) provided a reference for comparison in the current study (Table 1.1). Tsuchida *et al.* (2014) recorded TNF-α mean ± SD concentrations in SF of 4.00 ± 20.00pg.mL^{-1} in the KOA patient group (*n*=27) and 0.00 ± 0.00pg.mL^{-1} in healthy SF of the healthy volunteer group (*n*=20). In the current study, only 1 patient (*n*=18) had detectable concentrations of TNF-α in SF. The detectable TNF-α mean ± SD concentration (duplicate replicates) was observed in P7 with 25.08 ± 0.91pg.mL^{-1}. This indicated that TNF-α may have been a major contributor to the inflammatory destructive process in the knee joint of P7.

4.6 Analysis of IFN-γ in the study

In the current study, the mean ± SD concentration for the patient group (*n*=20) was 0.95 ± 2.72pg.mL^{-1} and 30.86 ± 90.69pg.mL^{-1} for the healthy volunteer group (*n*=18). Serum IFN-γ were only detectable in 4 patients (Figure 3.15a) so concentrations greater than 0.00 pg.mL^{-1} were considered "high". The highest mean ± SD concentrations of serum IFN-γ were reported in P8 with 4.68 ± 0.10pg.mL^{-1} and P9 with 11.52 ± 0.83pg.mL^{-1}. These patients may have had a form of KOA whereby IFN-γ was involved in the inflammatory cytokine cascade. The highest mean ± SD concentration of serum IFN-γ reported in the healthy volunteer group was V12b with 385.04 ± 31.99pg.mL^{-1} (Figure 3.15b). The elevated IFN-γ concentration in V12b may have indicated the presence of an autoimmune disease. Diseases associated with high IFN-γ concentrations include multiple scelerosis (Brandao *et al.*, 2005), inflammatory bowel disease (Charles *et al*., 2014) and RA (Chung *et al.*, 2011).

There was not a significant difference in IFN-γ serum concentrations between the patient and healthy volunteer groups (P>0.05). The data suggested that serum IFN-γ may not be a suitable biomarker for KOA. In the current study, high IFN-γ mean ± SD concentrations were reported in 3 patients (Figure 3.16) that participated in the study (P7 with 54.90 ± 2.91pg.mL^{-1}, P11 with 14.55 ± 13.78pg.mL^{-1} and P14 with 54.90 ± 2.91pg.mL^{-1}). This indicated that IFN-γ was a major contributor to the inflammatory destructive process in the knee joint of P7. The IFN-γ SF concentration determined in the healthy volunteer study group by Tsuchida *et al*. (2014) provided a reference for comparison in the current study (Table 1.1). Tsuchida *et al.* (2014) reported SF IFN-γ mean ± SD concentrations of 51.00 ± 69.00pg.mL^{-1} in the KOA patient group (*n*=27) and 47.00 ± 17.00pg.mL^{-1} in healthy SF of the healthy volunteer group (*n*=20).

4.7 Analysis of IL-13 in the study

In the current study, the inclusion of a healthy volunteer group determined the normal serum IL-13 concentrations. The serum IL-13 mean ± SD concentration reported were 15.83 ± 43.49pg.mL^{-1} for the patient group (*n*=20) and 30.68 ± 99.29pg.mL^{-1} for the healthy volunteer group (*n*=18). Serum IL-13 above 0.00pg.mL^{-1} were considered "high" as IL-13 was only detectable in 5 patients. The serum IL-13 mean ± SD concentrations in the patient group ranged from 7.35 ± 0.67pg.mL^{-1} (P30) to 185.73 ± 21.14pg.mL^{-1} (P7) and in the healthy volunteer group detectable values ranged from 7.41 ± 0.55 pg.mL^{-1} to 410.21 ± 17.94pg.mL^{-1} (Figure 3.18). V12b presented with the highest serum IL-13 concentration which may have indicated autoimmune disease. Other patients with high mean ± SD concentrations of serum IL-13 included P4 with 70.34 ± 0.34pg.mL^{-1}, P11 with 30.74 ± 2.33pg.mL^{-1} and P15 with 22.49 ± 3.47pg.mL^{-1} (Figure 3.18). These patients may have had a form of KOA whereby IL-13 was involved in the inflammatory cytokine cascade. Silosi *et al.* (2016) reported the mean IL-13 concentration of 4.80pg.mL^{-1} in the serum of healthy volunteers (*n*=28). There was not a significant difference in IL-13 serum concentrations between the patient and healthy volunteer groups ($P>0.05$). The data suggested that serum IL-13 may not have been a suitable biomarker for KOA.

In the current study, high SF IL-13 mean ± SD concentrations were reported in 3 patients (Figure 3.19) that participated in the study (P7 with 131.35 ± 12.95pg.mL^{-1}, P11 with 25.83 ± 0.84pg.mL^{-1} and P15 with 4.27 ± 0.80pg.mL^{-1}) (Figure 3.19). This indicated that IL-13 was a major contributor to the inflammatory destructive process in the knee joint of P7. The analysis of matched serum and SF samples for IL-13 provided a clearer depiction of the cytokine cascade in P7 and P11. High IL-13 concentrations have been linked to autoimmune diseases such as RA (Silosi *et al.*, 2016) and irritable bowel syndrome (IBS) (Charles *et al.*, 2014). The IL-13 SF concentration determined in the healthy volunteer study group by Tsuchida *et al.* (2014) provided a reference for comparison in the current study (Table 1.1). Tsuchida *et al.* (2014) reported SF IL-13 mean ± SD concentrations of 18.00 ± 40.00pg.mL^{-1} in the KOA patient group (*n*=27) and 1.00 ± 2.00pg.mL^{-1} in SF of the healthy volunteer group (*n*=20).

4.8 Cytokine profiles for individuals in the study

The 2 patients in the current study (P7 and P11) presented the highest concentrations of the cytokines in their SF samples (Table 3.1). This outcome suggested that the form of KOA in P7 and P11 was characterised by a cytokine imbalance with significantly high SF concentrations of IL-6 (P7 presented with an IL-6 mean ± SD concentration of 2258.39 ± 10.10pg.mL^{-1}, while P11 presented with 2021.82 ± 26.69pg.mL^{-1}). The high concentrations of IL-6 in P7 and P11 suggested that they suffer from an inflammatory form of KOA. The 3 healthy volunteers (V2b, V12b and V25b) presented the highest concentrations of cytokines in their serum samples (Table 3.1). This outcome suggested the healthy volunteers (V2b, V12b and V25b) may be high risk candidates for autoimmune disease as they presented with a cytokine imbalance with high serum concentrations of IFN-γ (V2b presented with a mean ± SD IFN-γ concentration of 38.85 ± 0.70pg.mL^{-1}, V12b presented with 385.04 ± 31.99pg.mL^{-1} and V25b presented with 78.60 ± 9.48pg.mL^{-1}) and IL-13 (V2b presented with a mean ± SD IL-13 concentration of 127.11 ± 11.60pg.mL^{-1}, V12b presented with 410.21 ± 17.94pg.mL^{-1} and V25b presented with 7.41 ± 0.55pg.mL^{-1}).

High concentrations of the two cytokines (IFN-γ and IL-13) have been associated with autoimmune diseases such as IBS (Charles *et al.*, 2013) and RA (Silosi *et al.*, 2016; Chung *et al.*, 2011). The cytokine concentration of IFN-γ in the serum samples of the participant with the study code, V2, was significantly difference at 2 time-point blood collections in the study, “a” and “b”. This indicted that this cytokines be affected by circadian rhythms. The cytokine concentration of IL-13 in the serum samples of V2 was similar at the 2 time-point blood collections in the study, “a” and “b”. This indicted that this cytokines may not be affected by circadian rhythms, although further investigation should be explored using a larger study group. The serum IL-6 mean concentrations were higher at time-point “a” (mean ± SD of 1.97 ± 0.35pg.mL^{-1}) than time-point “b” (mean ± SD of 1.97 ± 0.35pg.mL^{-1}); a significant difference of 0.50pg.mL^{-1}. The difference in serum IL-6 concentrations between the 2 time-points may have been due to the fact this cytokine is affected by circadian rhythms (Mabey and Honsawek, 2015).

4.9 Gelatin zymography

Gupta *et al.* (2007) determined the 125kDa band to be the NGAL MMP-9 complex in SF samples from OA patients. In the current study, the bands detected between the 75kDa and 100kDa markers represented the 82kDa active MMP-9 and the 92kDa pro MMP-9 (Figure 3.23, Figure 3.24 and Figure 3.25) (Protocol Place, 2013). Total MMP-9 consisted of the band containing both the active and pro forms of MMP-9 (82kDA and 92kDa bands). Gelatin zymograms that were analysed using Image J analysis (Figure 3.23 and Figure 3.24) had total MMP-9 concentrations that were higher in KOA patients than the healthy volunteer group. This may have indicated that high total MMP-9 concentrations was linked to KOA, although further investigation was required. Although, many of the gelatin zymograms produced were not suitable for ImageJ analysis, visually the 125kDa band was observed the patient samples (Figure 3.25). This band has been linked to OA in SF samples (Gupta *et al.*, 2007). The current study demonstrated the presence of the 125kDa band in plasma using gelatin zymography may have indicated OA, which may be useful for the diagnosis of the condition.

Chapter 5: Conclusions and recommendations

The current study was a success as the aims and objectives were met. However, many limitations and flaws must be considered in the research project. The limitations and flaws in the study design were as follows: the health status of the participants in the healthy volunteer group was not assessed by a general practitioner prior to participation; healthy SF was unavailable therefore healthy SF was not included in testing; and the technique used for cytokine quantification (the ELISA assay) is affected by false positives and the hook effect. The analytical technique, LC-MS/MS may provide a good alternative to the ELISA assay as it is unaffected by false positives and has better multiplexing capability, however, this technique is more expensive than the ELISA assay (Ademowo *et al*., 2013). Gelatin zymography was useful to analyse MMPs but had many limitations that must be considered. There was considerable variation between gelatin zymograms which resulted in incomparable MMP concentrations in plasma samples from KOA patients and healthy volunteers. To obtain less variability between gels, gelatin zymography kits should be purchased from commercial companies.

Based on data presented in the current study, the most suitable cytokines to be used as biomarkers for KOA in serum samples were IL-6 and IL-10 as a significant difference was reported between the KOA patient group and the healthy volunteer group for these cytokines. Serum IL-17A, TNF-α, IFN-γ and IL-13 may be unsuitable as biomarkers for KOA as no significant differences were observed between the KOA patient group and the healthy volunteer group. The analysis of IL-17A, TNF-α, IFN-γ and IL-13, IL-6 and IL-10 in SF samples provided a clearer depiction of the inflammatory state of KOA in the knee joint of patients. The 2 KOA patients in the current study, P7 and P11, may have suffered from an inflammatory form of KOA where the SF in the knee joint was characterised by high concentrations of IL-6, IL-10, IL-13 and IFN-γ (Table 3.1). The cytokines, IFN-γ and IL-13, were present at high concentrations in the serum of 3 healthy volunteers (Table 3.1) in the current study which may have indicated early arthritis or autoimmune disease or the inflammation may have been due to inflammation from another source such as gum disease or periodontitis. Further investigation into the use of serum IL-6 and IL-10 as biomarkers for KOA should be explored.

In the current study, the serum IL-6 mean concentrations were higher at time-point collection "a" than time-point collection "b" with a significant difference of 0.50pg.mL^{-1}. The difference in serum IL-6 concentrations between the 2 time-points may have been due to the fact this cytokine is affected by circadian rhythms (Mabey and Honsawek, 2015). The fact that IL-6 concentrations are affected by circadian rhythms may be problematic when interpreting data so more stringent controls should be implemented in future studies to account for this. The concentrations of total MMP-9 in plasma samples from KOA patients were higher than the healthy volunteer group (Figure 3.23 and Figure 3.24). MMP-2 was undetectable in many of the samples analysed so this was not suitable for comparison using ImageJ analysis. The current study demonstrated the presence of this 125kDa band in plasma using gelatin zymography may indicate OA, which may be useful for the diagnosis of the condition.

In future studies, the following is recommended: participants should be sex- and age-matched; the markers or cytokines analysed in the study should be correlated with the degree and/ or severity of symptoms encountered in KOA; healthy SF samples should be included to compare to cytokine concentrations in the patient group; and synovial tissue should be included as a specimen type to obtain a clearer depiction of the cytokine profile in the knee of KOA patients. Healthy SF samples should be obtained from donors within 24 hours of post-mortem as described by Tsuchida *et al.* (2014). The inflammatory response encountered may be associated with average daily lifestyle activities in both the patient and healthy groups so this could be explored in future studies (Ertek and Cicero, 2012). The clinical benefit from the current research project is the potential for targeted treatment in KOA patients and the development of an early diagnosis detection system for autoimmune disease by screening cytokines in the healthy population. For example, targeted treatment in KOA patients with high IL-6 concentrations might be achieved. High IL-6 concentrations might suggest that KOA patients would respond well to drugs that target this cytokine such as the biologic drug, Tocilizumab (a monoclonal antibody) (O'Reilly *et al.,* 2013). This drug acts by blocking the IL-6 receptor inhibiting inflammation and is used to treat diseases such as RA and JIA (O'Reilly *et al.,* 2013).

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Appendices

Appendix I: Consent form for participation in the study

The human participant informed consent form (attached below) was completed by students and staff at the Institute of Technology Carlow prior to participation in the study. All consent forms were submitted to the Ethics Boards at the Institute of Technology Carlow and AutEven Hospital.

CONSENT FORM: **Human Participants**

RESEARCH - INFORMED CONSENT FORM

INSTITUTE of TECHNOLOGY CARLOW

I. Project Title: Can assessment of synovial fluid and blood samples for pro- and anti-inflammatory markers help predict the type of arthritis patients will develop?

II. Introduction to the study:

In this project, synovial fluid samples will be taken from patients presenting with synovial fluid effusions at a local GP surgery. (Permission has been obtained from the lead GP). During inflammation, various pro-inflammatory cytokines are produced. Inflammation is divided into acute inflammation and chronic inflammation, which occurs over longer times and can persist over weeks, months and years. Acute inflammation begins immediately after tissue injury, damage or trauma.

If acute inflammation is not resolved, the inflammation may progress into a longer term chronic phase. Rheumatoid arthritis (RA) is characterized by varying levels of inflammation in synovial tissue.

The varying levels of inflammation in RA are reflected by alterations in the acute-phase response, which is induced by inflammation, infection and tissue damage.

Cytokines are released by living cells of the host. Pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), Interleukin-8 (IL-8), Interleukin-12 (IL-12) play an important role in starting and continuing inflammation and destructive processes in the rheumatoid joint. Prostaglandin E_2 (PGE_2) and nitric Oxide (NO) are regulated by cytokines and promote inflammation and play a role in the destructive mechanisms, matrix metalloproteinases production (MMPs) and Tissue Inhibitors of Metalloproteinases (TIMPs) in the joint. There are also some anti-inflammatory cytokines that act to dampen down the inflammatory response by inhibition of the production of pro-inflammatory cytokines. These include Interleukin-4, 10 and 13.

III. Purpose of the study:

The aim of this project is to investigate the presence and balance of these pro- and anti-inflammatory cytokines and the presence or absence of MMPs in synovial fluid. The student would then carry out a retrospective analysis and correlate results with consultant diagnosis (based on their physical and investigative findings using MRI etc.).

IV. This research study will take place at the Institute of Technology, Carlow.

V. This is what will happen during the research day

VI. Sometimes there are problems associated with this type of study. These are:

Getting sufficient paired Blood and synovial samples. However, if this is not possible to attain paired blood and synovial fluid samples, blood samples will be used.

VII. Their maybe benefits to me from this research.

Getting papers published in scientific journals.

VIII. My confidentiality will be guarded. IT Carlow will make reasonable efforts to protect the information about me and my part in this study and no identifying data will be published. This will be achieved by assigning me an ID number against which all data will be stored. Details linking my ID number and name will not be stored with the data. The results of the study maybe published and used in further studies.

IX. If you have any questions about the study, please feel free to ask:

- Dr Rosemary O'Hara, IT Carlow. 059-9175517, rosemary.ohara@itcarlow.ie
- Ms Paula Rankin, IT Carlow. 059-9175540, paula.rankin@itcarlow.ie
- Dr Gerard Moran, Tullow Street, Carlow. 059-9130770.

Taking part in this study is my decision. If I do agree to take part, I may withdraw at any point including during the interview. There will be no penalty if I withdraw before I have completed the study.

XI. Signature: _____

I have read and understood the information in this form. My questions and concerns have been answered by the researchers, and I have a copy of this consent form. Therefore, I consent to take part in this research project entitled: "Establishing best practice in tailoring lifestyle interventions for obese men in a primary care setting".

Signature: _____ Date: _____

Witness: _____ Witness: _____

Signature Printed name

Appendix D.

Blood Specimen Consent Form

Blood/Synovial Fluid Specimen Consent Form

INSTITUTE of TECHNOLOGY CARLOW

Dear Participant

You are asked to participate in a Research Study entitled:

Can assessment of synovial fluid and blood samples for pro- and anti-inflammatory markers help predict the type of arthritis patients will develop?

1. You are requested to give a single anonymous blood sample (10 ml) and synovial fluid sample (10ml) to help this research which is aimed at determining the levels of pro- and anti- inflammatory markers, which may help predict which type of arthritis a patient may develop.

2. **There is no appreciable risk associated with giving this sample. Your decision to participate is entirely your own (voluntary) and you may withdraw consent at any time.** Such a decision in no way affects your position as a student or staff member at IT Carlow. You will receive a copy of the consent form.

3. This study has been approved by the Research Ethics Committee of the Institute and is part-funded by Presidents Research Fellowship, IT Carlow.....
    a. Dr Rosemary O'Hara, IT Carlow. 059-9175517, rosemary.ohara@itcarlow.ie
    b. Ms Paula Rankin, IT Carlow. 059-9175540, paula.rankin@itcarlow.ie
    c. Dr Gerard Moran, Tullow Street, Carlow. 059-9130770.
    d. Mr. Laurence O'Neill, IT Carlow.

Arthritis is due to several factors, one of these factors is the presence of cytokines. These are small glycoproteins that are produced as a result of inflammation which occurs in arthritis. Some cytokines may promote inflammation and other cytokines may dampen down inflammation. By determining the amounts of different cytokines present in blood or synovial fluid (found in swollen and painful knee joints) it may be possible to predict which patients may go on to develop different types of arthritis.

(INCLUDE DETAILS OF RESEARCHERS INVOLVED AND THE PURPOSE AND A BRIEF DESCRIPTION OF THE PROJECT IN LAYMANS TERMS HERE)

4. It should take no longer than 5 minutes to give the blood sample and synovial Fluid sample.

5. This study should help in the understanding of how various chemicals in the blood and/ or synovial fluid may help to predict if patients will develop a particular type of arthritis.

Thank you for taking the time to read this material and also for volunteering, should you decide to do so. The undersigned will be delighted to give you further information if required.

Name: Dr Rosemary O'Hara
Address: IT Carlow
Tel no.: 059-9175517.

Thank You.

Can assessment of synovial fluid and blood samples for pro- and anti-inflammatory markers help predict the type of arthritis patients will develop?

CONSENT FORM

Your signature:_____ Date: _____

Your name (PRINTED)

Witness signature:_____ Date: _____

Appendix II: Volunteer assessment form for participation in the study

The volunteer subject assessment form (attached below) was completed by students and staff at the Institute of Technology Carlow prior to participation in the study.

Voluntary Assessment Form

The Assessment Form is required for voluntary donations of blood for research purposes at the Institute of Technology Carlow

Volunteer Number: V1 etc.

Personal details

Please fill in the table as best you can

Date	
Name	
Address	
Age	
Sex	
Ethnicity	

Arthritis History

Please tick the relevant box if you suffer from this condition

Form	***Yes***	***No***
Arthritis (unknown type)		
Osteoarthritis		
Gout		
Childhood Arthritis		
Lupus/ "SLE"		
Rheumatoid Arthritis		
Ankylosing Spondylitis		
Osteoporosis		

Other Arthritis Condition (please specify):_____

Past Medical History

Do you or have you ever had any of the following conditions:

Condition	***Yes***	***No***
Psoriasis		
Cancer		

Smoking and Alcohol History

Please tick relevant box and answer question in comment box

Question	***Yes***	***No***	***Comment***
Do you smoke? How long ago?			
Do you drink alcohol? Number per week?			

Results of the Study

Question	***Yes***	***No***
Would you like to know the results of the testing done on your blood samples?		

Privacy

The investigator in the study will not disclose the names of volunteers and any other confidential information in the project. The researcher will only give the patient numbers in the thesis. Privacy is ensured by submitting all consent forms and voluntary assessment forms to the Principal Investigator of the study.

Patient's Name_____ Date_____ Principal Investigator Initials_____

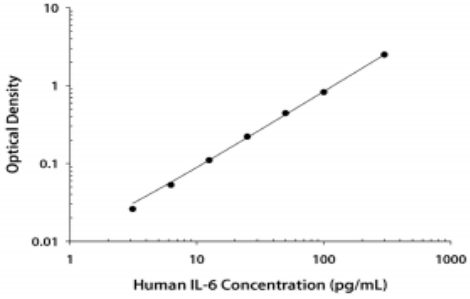
Appendix III: Technical sheet for the IL-6 R&D systems ELISA kit

For technical information on the R&D Systems IL-6 Quantikine precoated ELISA kit (catalogue #: D6050), the information booklet provided with the kit was consulted. This booklet contained information on the method to follow and the technical information as presented below.

Typical data

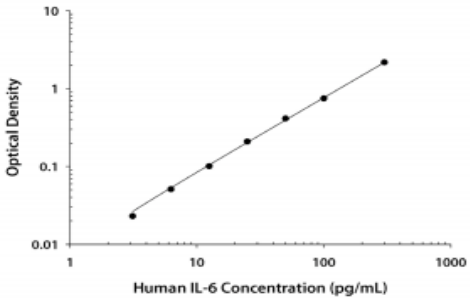
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.022 0.028	0.025	—
3.13	0.050 0.052	0.051	0.026
6.25	0.078 0.078	0.078	0.053
12.5	0.134 0.136	0.135	0.110
25	0.247 0.245	0.246	0.221
50	0.472 0.465	0.468	0.443
100	0.865 0.836	0.850	0.825
300	2.524 2.515	2.520	2.495

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.025 0.029	0.027	—
3.13	0.049 0.051	0.050	0.023
6.25	0.078 0.077	0.078	0.051
12.5	0.127 0.129	0.128	0.101
25	0.236 0.236	0.236	0.209
50	0.438 0.442	0.440	0.413
100	0.780 0.773	0.776	0.749
300	2.176 2.221	2.198	2.171

Precision

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	15.8	95.6	179	16.4	98.8	188
Standard deviation	0.7	3.0	3.1	0.6	2.5	3.7
CV (%)	4.4	3.1	1.7	3.7	2.5	2.0

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	16.8	97.7	186	17.2	101	191
Standard deviation	0.7	1.6	3.8	1.1	3.3	7.2
CV (%)	4.2	1.6	2.0	6.4	3.3	3.8

Recovery

The recovery of human IL-6 spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=5)	98	94-103%
Serum (n=5)	93	86- 99%
EDTA plasma (n=5)	95	84-101%
Heparin plasma (n=5)	90	88-98%
Citrate plasma (n=5)	91	82-95%

Sensitivity

The minimum detectable dose (MDD) of human IL-6 is typically less than 0.70 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

Linearity

To assess the linearity of the assay, samples were spiked with high concentrations of human IL-6 in various matrices and diluted with the appropriate calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Citrate plasma (n=4)
1:2	Average % of Expected	99	97	101	103	101
	Range (%)	96-101	92-100	98-105	96-109	96-106
1:4	Average % of Expected	100	101	104	106	105
	Range (%)	93-110	93-107	97-110	97-113	101-109
1:8	Average % of Expected	96	102	100	104	106
	Range (%)	92-100	96-108	86-112	93-111	101-111
1:16	Average % of Expected	94	103	99	105	101
	Range (%)	83-108	93-111	90-110	99-107	90-114

Calibration

This immunoassay is calibrated against highly purified *E. coli*-expressed recombinant human IL-6 produced at R&D Systems®. The NIBSC/WHO 1st International Standard for IL-6 (89/548), which was intended as a potency standard, was evaluated in this kit. The NIBSC/WHO standard is a CHO cell-derived recombinant human IL-6.

The dose response curve of the International Standard (89/548) parallels the Quantikine® standard curve. To convert sample values obtained with the Quantikine® Human IL-6 kit to approximate NIBSC 89/548 units, use the equation below.

NIBSC (89/548) approximate value (IU/mL)=0.131 x Quantikine® Human IL-6 value (pg/mL)

Sample values

Serum/Plasma - Forty serum and plasma samples from apparently healthy volunteers were evaluated for the presence of human IL-6 in this assay. Thirty-three samples measured less than the lowest standard, 3.13 pg/mL. Seven samples measured between 3.13 and 12.5 pg/mL. No medical histories were available for the donors used in this study.

Cell Culture Supernates - Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate and stimulated for 1, 3, and 5 days with 10 µg/mL PHA. Aliquots of the culture supernates were removed on days 1, 3, and 5 and assayed for levels of human IL-6.

Condition	Day 1 (pg/mL)	Day 3 (pg/mL)	Day 5 (pg/mL)
Unstimulated	575	311	660
Stimulated	17,130	17,520	16,340

Specificity

This assay recognizes natural and recombinant human IL-6.

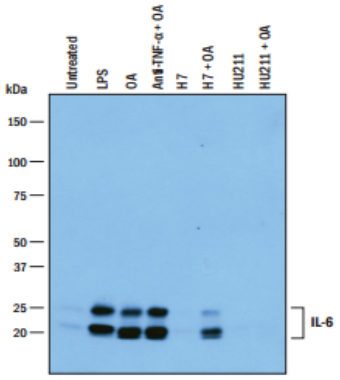
The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5T and at 100 ng/mL in Calibrator Diluent RD6F and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human IL-6 control prepared in Calibrator Diluent RD5T and 100 ng/mL in a mid-range IL-6 control prepared in Calibrator Diluent RD6F were assayed for interference. No significant cross-reactivity or interference was observed.

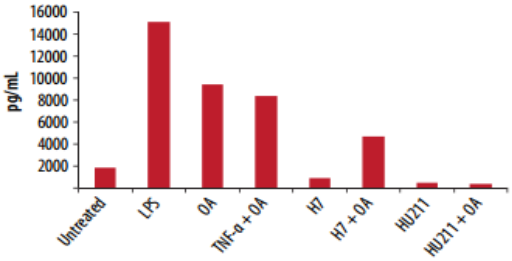
Recombinant human:
CNTF
G-CSF
GM-CSF
gp130
IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-6 Rα
IL-6 Rα/gp130
IL-7
IL-8
IL-11
IL-12
LIF
LIF R
OSM
TNF-α
TNF-β

Recombinant mouse:
GM-CSF
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-11
IL-12

Recombinant rat:
CNTF

Natural proteins:
bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF-β1
porcine TGF-β1.2
porcine TGF-β2





Monocytes were prepared from human PBMCs by adherence to plastic. Adherent monocytes were washed, replated, and allowed to rest for 24 hours. Pretreatments were for 30 minutes: neutralizing anti-human TNF-α (R&D Systems®, Catalog # MAB610) at 5 µg/mL, H7 serine kinase inhibitor (Tocris, Catalog # 0542) at 10 µM, or HU211 NFκB inhibitor (Tocris, Catalog # 2861) at 10 µM. Following the pretreatment, 500 ng/mL LPS or 30 ng/mL okadaic acid (OA, Tocris, Catalog # 1136) was added for 20 hours as indicated. Conditioned media was tested in the Quantikine® ELISA, resolved by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with the detection antibody used in this kit. The immunoprecipitation/ Western Blot shows direct correlation with the ELISA value for these samples.

MANUFACTURED AND DISTRIBUTED BY:

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614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
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E-MAIL: info@RnDSystemsChina.com.cn

Appendix IV: Technical sheet for the IL-6 eBioscience Affymetrix ELISA kit

For technical information on the eBioscience Affymetrix IL-6 Platinum precoated ELISA kit (catalogue #: BMS213/2CE), the information booklet provided with the kit was consulted. This booklet contained information on the method to follow and the technical information as presented below.

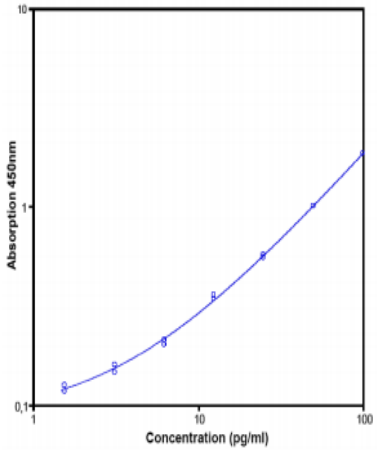
Typical data

Typical data using the human IL-6 ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human IL-6 Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	100.00	1.848	1.851	0.2
		1.854		
2	50.00	1.005	1.004	0.2
		1.002		
3	25.00	0.553	0.562	2.1
		0.570		
4	12.50	0.355	0.349	2.4
		0.343		
5	6.25	0.201	0.207	3.8
		0.212		
6	3.13	0.146	0.152	5.6
		0.158		
7	1.56	0.116	0.121	5.3
		0.125		
Blank	0.00	0.075	0.081	
		0.086		

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

Representative standard curve for human IL-6 ELISA. Human IL-6 was diluted in serial 2-fold steps in Assay Buffer (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Sensitivity

The limit of detection of human IL-6 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.92 pg/ml (mean of 6 independent assays).

Reproducibility

1. Intra-assay

Reproducibility within the assay was evaluated in two independent experiments. Each assay was carried out with six replicates of eight serum samples containing different concentrations of human IL-6. Two standard curves were run on each plate. Data below show the mean human IL-6 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 3.4%.

Table 3
The mean human IL-6 concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human IL-6 Concentration (pg/ml)	Coefficient of Variation (%)
1	1	40.7	7.8
	2	42.2	1.6
2	1	40.1	4.1
	2	40.1	2.6
3	1	43.2	1.1
	2	41.7	3.5
4	1	65.6	2.3
	2	65.4	4.6
5	1	47.2	1.6
	2	48.0	2.1
6	1	34.1	2.5
	2	37.8	5.4
7	1	27.3	0.2
	2	35.2	7.7
8	1	37.8	4.1
	2	42.6	2.4

2. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 2 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human IL-6. 2 standard curves were run on each plate. Data below show the mean human IL-6 concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 5.2%.

Table 4

The mean human IL-6 concentration and the coefficient of variation of each sample

Sample	Mean Human IL-6 Concentration (pg/ml)	Coefficient of Variation (%)
1	41.5	2.6
2	40.1	0.0
3	42.5	4.4
4	65.5	0.2
5	47.6	1.2
6	35.9	7.3
7	31.3	17.8
8	40.2	8.4

Spike recovery

The spike recovery was evaluated by spiking 4 levels of human IL-6 into serum. Recoveries were determined in 2 independent experiments with 8 replicates each.

The unspiked serum was used as blank in these experiments.

The recovery ranged from 78% to 105% with an overall mean recovery of 88%.

Dilution parallelism

Serum samples with different levels of human IL-6 were analysed at serial 2 fold dilutions with 4 replicates each.

The recovery ranged from 98% to 111% with an overall recovery of 105% (see Table 5).

Table 5

Sample	Dilution	Expected Human IL-6 Concentration (pg/ml)	Observed Human IL-6 Concentration (pg/ml)	Recovery of Expected Concentration (%)
1	1:2	-	46.4	-
	1:4	23.2	22.7	98
	1:8	11.6	11.8	102
2	1:2	-	95.0	-
	1:4	47.5	50.3	106
	1:8	23.8	23.4	99
3	1:2	-	51.9	-
	1:4	26.0	28.8	111
	1:8	13.0	14.4	111

Comparison of serum and plasma

From two individuals, serum as well as EDTA, citrate and heparin plasma obtained at the same time point were evaluated. Human IL-6 concentrations were not significantly different and therefore all these body fluids are suitable for the assay. It is nevertheless highly recommended to assure the uniformity of blood preparations.

Specificity

The interference of circulating factors of the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into a human IL-6 positive serum.

There was no crossreactivity detected.

Expected values

A panel of samples from randomly selected apparently healthy donors (males and females) was tested for human IL-6.

The levels measured may vary with the sample collection used. For detected human IL-6 levels see Table 6.

Table 6

Sample Matrix	Number of Samples Evaluated	Range (pg/ml)	% Detectable	Mean of Detectable (pg/ml)
Serum	40	nd *- 12.7	47.5	5.8
Plasma (EDTA)	40	nd *- 13.0	17.5	6.4
Plasma (Citrate)	40	nd *- 6.6	2.5	6.6
Plasma (Heparin)	40	nd *- 6.5	30.0	5.0

* n.d. = non-detectable, samples measured below the lowest standard point are considered to be non-detectable.

Calibration

The immunoassay is calibrated with highly purified recombinant human IL-6 which has been evaluated against the international Reference Standard NIBSC 89/548 and has been shown to be equivalent.

NIBSC 89/548 is quantitated in International Units (IU), 1IU corresponding to 10 pg human IL-6.

Branding

Starting in early 2017, Affymetrix and eBioscience products became branded under Thermo Scientific (Biosystems and Invitrogen) with new packaging and logos implemented. The Affymetrix eBioscience ELISA kits became branded under Invitrogen.

Appendix V: Technical sheet for the IFN-γ eBioscience Affymetrix ELISA kit

For technical information on the eBioscience Affymetrix IFN-γ Platinum precoated ELISA kit (catalogue #: BMS228), the information booklet provided with the kit was consulted. This booklet contained information on the method to follow and the technical information as presented below.

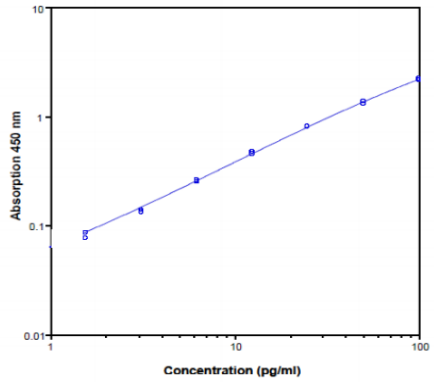
Typical data

Typical data using the human IFN gamma ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human IFN gamma Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	100.00	2.210	2.177	2.2
		2.143		
2	50.0	1.307	1.342	3.6
		1.376		
3	25.0	0.802	0.804	0.3
		0.805		
4	12.5	0.450	0.463	3.8
		0.475		
5	6.3	0.257	0.254	2.0
		0.250		
6	3.1	0.139	0.136	3.7
		0.132		
7	1.6	0.085	0.081	7.0
		0.077		
Blank	0	0.027	0.027	0
		0.027		

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

Representative standard curve for human IFN gamma ELISA. Human IFN gamma was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Sensitivity

The limit of detection of human IFN gamma defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.99 pg/ml (mean of 6 independent assays).

Reproducibility

1. Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human IFN gamma. 2 standard curves were run on each plate. Data below show the mean human IFN gamma concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 4.5%.

Table 3

The mean human IFN gamma concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human IFN gamma Concentration (pg/ml)	Coefficient of Variation (%)
1	1	173	2.5
	2	184	7.3
	3	160	2.1
2	1	216	3.0
	2	220	5.4
	3	212	1.7
3	1	101	2.0
	2	112	2.4
	3	100	0.3
4	1	290	1.6
	2	300	3.7
	3	277	0.5
5	1	18.0	4.9
	2	18.5	7.8
	3	18.7	6.7
6	1	27.9	3.1
	2	31.1	8.0
	3	27.4	6.7
7	1	75.4	7.7
	2	69.5	5.3
	3	61.4	4.6
8	1	9.6	2.9
	2	11.0	7.2
	3	9.7	10.7

2. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human IFN gamma. 2 standard curves were run on each plate. Data below show the mean human IFN gamma concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 5.7%.

Table 4
The mean human IFN gamma concentration and the coefficient of variation of each sample

Sample	Mean Human IFN gamma Concentration (pg/ml)	Coefficient of Variation (%)
1	172.0	7.2
2	216.0	1.8
3	104.0	5.9
4	289.0	3.9
5	18.4	2.0
6	28.8	6.8
7	68.8	10.3
8	10.1	7.8

Spike recovery

The spike recovery was evaluated by spiking 4 levels of human IFN gamma into a pooled normal serum sample. Recoveries were determined in 3 independent experiments with 8 replicates each. The unspiked serum was used as blank in these experiments. The recovery ranged from 88% to 112% with an overall mean recovery of 97%.

Dilution parallelism

4 serum samples with different levels of human IFN gamma were analysed at serial 2 fold dilutions with 4 replicates each.
The recovery ranged from 86% to 114% with an overall recovery of 99% (see Table 5).

Table 5

Sample	Dilution	Expected Human IFN gamma Concentration (pg/ml)	Observed Human IFN gamma Concentration (pg/ml)	Recovery of Expected Human IFN gamma Concentration (%)
1	1:2	--	144.4	--
	1:4	72.2	67.0	93
	1:8	36.1	35.6	99
	1:16	18.0	15.8	87
2	1:2	--	161.7	--
	1:4	80.8	81.2	101
	1:8	40.4	44.1	109
	1:16	20.2	23.0	114
3	1:2	--	100.4	--
	1:4	50.2	44.9	90
	1:8	25.1	23.2	93
	1:16	12.5	10.7	86
4	1:2	--	261.5	--
	1:4	130.8	140.5	108
	1:8	65.4	69.4	106
	1:16	32.7	32.5	99

Comparison of serum and plasma

From two individuals, serum as well as EDTA, citrate, and heparin plasma obtained at the same time point were evaluated. Human IFN gamma concentrations were not significantly different and therefore all these body fluids are suitable for the assay. It is nevertheless highly recommended to assure the uniformity of blood preparations.

Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human IFN gamma positive serum. There was no crossreactivity detected.

Expected values

Panels of 40 serum as well as EDTA, citrate and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human IFN gamma.
Elevated human IFN gamma levels depend on the type of immunological disorder. The levels measured may vary with the sample collection used.
For detected human IFN gamma levels see Table 6.

Table 6

Sample Matrix	Number of Samples valuated	Range (pg/ml)	% Detectable	Mean of Detectable pg/ml)
Serum	40	nd *- 188.9	10.0	55.7
Plasma (EDTA)	40	nd *- 9.1	7.5	6.0
Plasma (Citrate)	40	nd *- 4.0	2.5	--
Plasma (Heparin)	40	nd *- 4.3	2.5	--

* n.d. = non-detectable, samples measured below the lowest standard point are considered to be non-detectable.

Calibration

The immunoassay is calibrated with highly purified recombinant human IFN gamma which has been evaluated against the international
Reference Standard NIBSC 82/587 and has been shown to be equivalent.
NIBSC 82/587 is quantitated in International Units (IU), 1IU corresponding to 50 pg human IFN gamma.

Branding

Starting in early 2017, Affymetrix and eBioscience products became branded under Thermo Scientific (Biosystems and Invitrogen) with new packaging and logos implemented. The Affymetrix eBioscience ELISA kits became branded under Invitrogen.

Appendix VI: Technical sheet for the IL-13 eBioscience Affymetrix ELISA kit

For technical information on the eBioscience Affymetrix IL-13 Platinum precoated ELISA kit (catalogue #: BMS231/3), the information booklet provided with the kit was consulted. This booklet contained information on the method to follow and the technical information as presented below.

Typical data

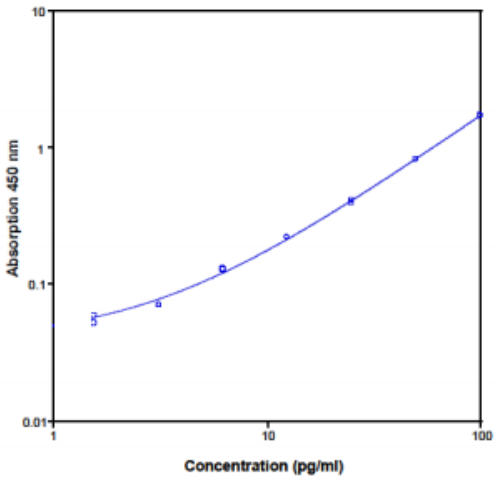
Typical data using the human IL-13 ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human IL-13 Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	100.0	1.693 1.659	1.676	1.4
2	50.0	0.807 0.814	0.811	0.6
3	25.0	0.403 0.388	0.396	2.7
4	12.5	0.216 0.218	0.217	0.7
5	6.3	0.126 0.127	0.127	0.6
6	3.1	0.07 0.07	0.07	0.0
7	1.6	0.058 0.051	0.055	9.1
Blank	0.0	0.019 0.021	0.020	5.0

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

Representative standard curve for human IL-13 ELISA. Human IL-13

was diluted in serial 2-fold steps in Assay Buffer (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Sensitivity

The limit of detection of human IL-13 defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.7 pg/ml (mean of 6 independent assays).

Reproducibility

1. Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human IL-13. 2 standard curves were run on each plate. Data below show the mean human IL-13 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 6.0%.

Table 3

The mean human IL-13 concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human IL-13 Concentration (pg/ml)	Coefficient of Variation (%)
1	1	103.54	2.0
	2	96.85	4.2
	3	89.21	3.2
2	1	71.18	8.7
	2	69.57	4.6
	3	75.89	11.1
3	1	57.62	10.0
	2	56.01	7.4
	3	57.12	7.0
4	1	44.55	8.8
	2	46.61	5.2
	3	47.00	7.2
5	1	33.20	7.6
	2	35.89	6.3
	3	35.54	11.8
6	1	31.04	6.1
	2	31.01	2.0
	3	28.16	2.0
7	1	22.86	7.7
	2	21.58	3.2
	3	21.63	4.2
8	1	16.43	6.2
	2	19.40	3.0
	3	18.48	5.8

2. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human IL-13. 2 standard curves were run on each plate. Data below show the mean human IL-13 concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 4.6%.

Table 4

The mean human IL-13 concentration and the coefficient of variation of each sample

Sample	Mean Human IL-13 Concentration (pg/ml)	Coefficient of Variation (%)
1	96.53	7.4
2	72.21	4.5
3	56.94	1.5
4	46.05	2.9
5	35.01	3.5
6	30.07	5.5
7	22.02	3.3
8	18.01	8.4

Spike recovery

The spike recovery was evaluated by spiking 3 levels of human IL-13 into serum. Recoveries were determined in 3 independent experiments with 6 replicates each.

The unspiked serum was used as blank in these experiments.

The recovery ranged from 93% to 110% with an overall mean recovery of 101%.

Dilution parallelism

4 serum samples with different levels of human IL-13 were analysed at serial 2 fold dilutions with 4 replicates each.

The recovery ranged from 93% to 123% with an overall recovery of 109% (see Table 5).

Table 5

Sample	Dilution	Expected Human IL-13 Concentration (pg/ml)	Observed Human IL-13 Concentration (pg/ml)	Recovery of Expected Human IL-13 Concentration (%)
1	1:2	-	105.5	-
	1:4	52.8	54.6	104
	1:8	27.3	28.6	105
	1:16	14.3	13.4	94
2	1:2	-	84.5	-
	1:4	42.2	46.8	110
	1:8	23.4	28.9	123
	1:16	14.4	16.5	115
3	1:2	-	40.7	-
	1:4	20.4	24.3	120
	1:8	12.2	13.2	108
	1:16	6.6	7.2	109
4	1:2	-	24.7	-
	1:4	12.3	12.9	105
	1:8	6.5	7.7	119
	1:16	3.9	3.6	93

Comparison of serum and plasma

Serum as well as EDTA, citrate and heparin plasma obtained from identical individuals at the same time point, were evaluated. Human IL-13 levels were not significantly different and therefore all these blood preparations are suitable for human IL-13 determinations. Nevertheless it is highly recommended to assure the uniformity of blood preparations.

Specificity

The assay detects both natural and recombinant human IL-13.

The interference of circulating factors of the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into a human IL-13 positive serum.

There was no crossreactivity detected.

Expected values

A panel of 24 serum samples from randomly selected apparently healthy donors (males and females) was tested for human IL-13.

The detected human IL-13 levels ranged between 0 and 44.4 pg/ml with a mean level of 8.2 pg/ml and a standard deviation of 12.1 pg/ml.

Calibration

The immunoassay is calibrated with highly purified recombinant human IL-13 which has been evaluated against the international Reference Standard NIBSC 94/622 and has been shown to be equivalent.

NIBSC 94/622 is quantitated in International Units (IU), 1IU corresponding to 1 ng human IL-13.

Branding

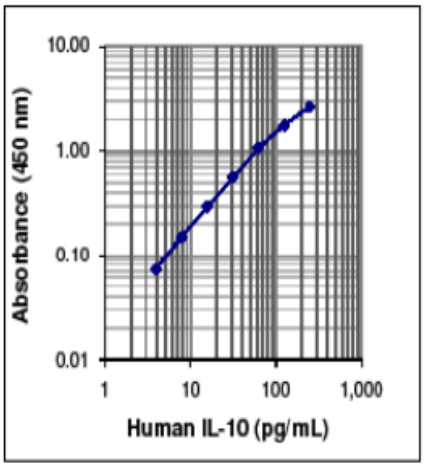
Starting in early 2017, Affymetrix and eBioscience products became branded under Thermo Scientific (Biosystems and Invitrogen) with new packaging and logos implemented. The Affymetrix eBioscience ELISA kits became branded under Invitrogen.

Appendix VII: Technical sheet for the IL-10 Biolegend ELISA kit

For technical information on the Biolegend IL-10 Max™ Deluxe uncoated ELISA kit (catalogue #: 430604), the information leaflet provided with the kit was consulted. This leaflet contained information on the method to follow and the technical information as presented below.

Typical Data

Standard Curve: This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance characteristics

Sensitivity: The expected minimum detectable concentration of IL-10 for this set is 2 pg/mL.

Specificity: No cross reactivity was observed when this kit was used to analyze multiple human, mouse and rat recombinant proteins.

Specimen collection and handling

Cell Culture Supernatant: If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at < -20°C. Avoid repeated freeze/thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 X *g*. Remove serum layer and assay immediately or store serum samples at < -20°C. Avoid repeated freeze/thaw cycles. Serum specimens should be clear and non-hemolyzed.

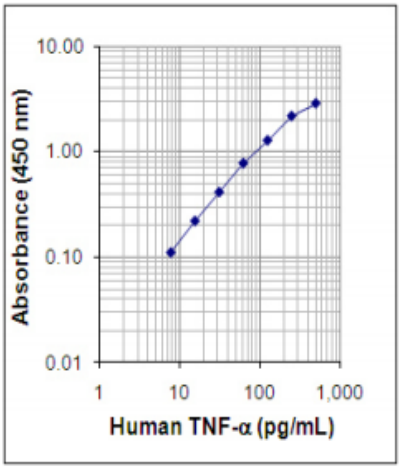
Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1,000 X *g* within 30 minutes of collection. Assay immediately or store plasma samples at < -20°C. Avoid repeated freeze/thaw cycles. Plasma specimens should be clear and non-hemolyzed.

Appendix VIII: Technical sheet for the TNF-α Biolegend ELISA kit

For technical information on the Biolegend TNF-α Max™ Deluxe uncoated ELISA kit (catalogue #: 430204), the information leaflet provided with the kit was consulted. This leaflet contained information on the method to follow and the technical information as presented below.

Typical Data

Standard Curve: This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance characteristics

Sensitivity: The expected minimum detectable concentration of TNF-α for this set is 2 pg/ml.

Specificity: No cross reactivity was observed when this kit was used to analyze multiple human, mouse and rat recombinant proteins.

Specimen collection and handling

Cell Culture Supernatant: If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at < -20°C. Avoid repeated freeze/thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 X *g*. Remove serum layer and assay immediately or store serum samples at < -20°C. Avoid repeated freeze/thaw cycles. Serum specimens should be clear and non-hemolyzed.

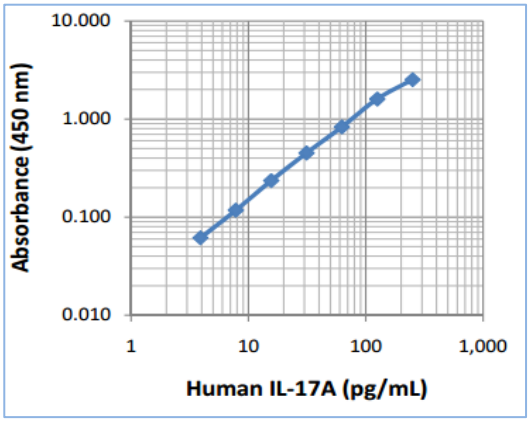
Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1,000 X *g* within 30 minutes of collection. Assay immediately or store plasma samples at < -20°C. Avoid repeated freeze/thaw cycles. Plasma specimens should be clear and non-hemolyzed.

Appendix IX: Technical sheet for IL-17A Biolegend ELISA kit

For technical information on the Biolegend IL-17A Max™ Deluxe uncoated ELISA kit (catalogue #: 433914), the information leaflet provided with the kit was consulted. This leaflet contained information on the method to follow and the technical information as presented below.

Typical Data

Standard Curve: This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance characteristics

Sensitivity: The expected minimum detectable concentration of IL-17A for this set is 2 pg/mL.

Specificity: No cross reactivity was observed when this kit was used to analyze 14 human and mouse recombinant cytokines/chemokines at up to 50 ng/ml.

Specimen collection and handling

Cell Culture Supernatant: If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at < -20°C. Avoid repeated freeze/thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 X *g*. Remove serum layer and assay immediately or store serum samples at < -20°C. Avoid repeated freeze/thaw cycles. Serum specimens should be clear and non-hemolyzed.

Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1,000 X *g* within 30 minutes of collection. Assay immediately or store plasma samples at < -20°C. Avoid repeated freeze/thaw cycles. Plasma specimens should be clear and non-hemolyzed.

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www.biolegend.com/support or
email: **techserv@biolegend.com**