Calprotectin (S100A8/A9) as a marker of inflammation and treatment monitoring in cases of Juvenile Idiopathic Arthritis in the Western Cape, South Africa

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Declaration

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Abstract

Background: Juvenile Idiopathic Arthritis (JIA) is a common rheumatic disease affecting children and is characterised by persistent inflammation of the joints. The socio-economic climate of South Africa can delay access to treatment to achieve remission. Joint inflammation is currently monitored through C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) measurements. Recent studies have shown that these markers may correlate less well with disease activity than calprotectin. Calprotectin is released by activated monocytes or macrophages at the site of joint damage and binds the TLR4 surface receptor. This protein has also been used to detect subclinical inflammation and may predict risk of relapse. This study aimed to compare standard markers of inflammation with calprotectin, and its relation to other inflammatory markers.

Methodology: Blood samples were collected from 22 consented JIA participants. Clinical information and history for each participant was obtained from patient files at Tygerberg Hospital. Monocyte distribution and phenotypic marker expression was investigated using whole blood for surface marker flow cytometry. Plasma levels of calprotectin and JIA associated inflammatory markers (including CCL2, CCL11, CD163, CXCL9, CCL3, CCL22, CD25, CXCL10, IL-1β, MIF, IL12, TNF-α and IFN-γ) were assessed by means of ELISA and Luminex™ multiplex assays. Routine CRP and ESR results were collected from the NHLS TrakCare database. For longitudinal follow up, blood samples were collected from the same cohort 6 months later and assays were repeated. A study database was created with all participant results, at both visits, to investigate relationships between calprotectin and JIA disease activity, as well as the effect of treatment over time.

Results: The majority (95%) of participants were already undergoing treatment. Calprotectin was within the normal range for children (127 – 1395 ng/mL) in 86% of baseline samples with a median of 628.6 ng/mL (IQR: 406.2 - 979.8). Only ESR changed significantly ($p=0.0079$) over time and showed the most evidence for changes in inflammation thereby inspiring analysis by stratification. To evaluate impact of disease phenotype (active vs. remission) and inflammatory state based on ESR expression (high vs. low ESR), participants were stratified into respective groups and compared. Expression of intermediate monocytes at baseline was higher than the expected range (2 - 10 %), with a median of 27 % (IQR: 9 - 43). This distribution is characteristic of inflammatory diseases. Calprotectin correlated significantly ($p<0.05$) with CRP and ESR at baseline in the returning, remission and high ESR analysis groups. Several notable cases displaying high calprotectin expression were linked to a relapse in disease.

Conclusions: Despite limitations, this study confirmed the predictive value of calprotectin in risk of disease relapse and the need for such a marker in the clinical setting to allow for a more tailored approach to treatment. The relationship between intermediate monocyte expansion, calprotectin and disease phenotype also needs to be examined further. Future studies which include treatment naïve participants would be beneficial in assessing the usefulness of calprotectin in monitoring response to treatment.

Opsomming

Agtergrond: Jeugdige idiopatiese artritis (JIA) is 'n algemene rumatieksiekte wat kinders affekteer en word gekenmerk deur aanhoudende inflammasie van die gewrigte. Die sosio-ekonomiese klimaat van Suid-Afrika kan toegang tot behandeling, om remissie te bewerkstellig, vertraag. Gewrigsontsteking word tans gemonitor deur C-reaktiewe proteïen (CRP) en eritrosiet sedimentasietempo (ESR) metings. Onlangse studies het getoon dat hierdie merkers nie so goed soos kalprotektien (KP) met siekte-aktiwiteit korreleer nie. KP word vrygestel deur geaktiveerde monosiete of makrofage op die plek van gewrigskade en bind die TLR4-oppervlakreseptor. Hierdie proteïen is ook gebruik om subkliniese inflammasie op te spoor en kan die risiko van terugval voorspel. Hierdie studie het ten doel gehad om standaardmerkers van inflammasie met KP te vergelyk, en dié se verband met ander inflammatoriese merkers.

Metodes: Bloedmonsters is van 22 ingestemde JIA-deelnemers ingesamel. Kliniese inligting is verkry uit pasiëntlêers te Tygerberg Hospitaal. Monosietverspreiding en fenotipiese merkeruitdrukking is ondersoek deur gebruik van volbloed vir vloeisitometrie. Plasmavlakke van KP en JIA-geassosieerde inflammatoriese merkers (insluitend CCL2, CCL11, CD163, CXCL9, CCL3, CCL22, CD25, CXCL10, IL-1β, MIF, IL12, TNF-α en IFN-γ) is deur middel van ELISA en Luminex™ multiplekstoetse geassesseer. Roetine CRP en ESR resultate is versamel van die NHLS TrakCare databasis. Vir opvolg, is bloedmonsters van dieselfde kohort 6 maande later versamel en toetse is herhaal. 'n Databasis is geskep met alle deelnemers' resultate, by beide besoeke, om verwantskappe tussen KP en JIA-siekteaktiwiteit te ondersoek, asook die effek van die behandeling.

Resultate: Die meerderheid (95%) van die deelnemers het reeds behandeling ondergaan. KP was binne die normale omvang vir kinders (127 – 1395 ng/mL) in 86% van basislynmonsters met 'n mediaan van 628.6 ng/mL (IQR: 406.2 - 979.8). Slegs ESR het beduidend verander (p=0.0079) met verloop van tyd en het die meeste bewyse getoon vir veranderinge in inflammasie en sodoende analise deur stratifikasie geïnspireer. Om die impak van siekte fenotipe (aktiewe vs. remissie) en inflammatoriese toestand te evalueer gebaseer op ESR uitdrukking (hoë vs. lae ESR), is deelnemers gestratifiseer en vergelyk. Uitdrukking van intermediêre monosiete by basislyn was hoër as die verwagte reeks (2 - 10 %), met 'n mediaan van 27 % (IQR: 9 - 43). Hierdie verspreiding is kenmerkend van inflammatoriese siektes. KP het betekenisvol ($p<0.05$) gekorreleer met CRP en ESR by basislyn in die terugkeer, remissie en hoë ESR analise groepe. Verskeie noemenswaardige gevalle wat hoë KP uitdrukking toon, is gekoppel aan 'n terugval in siekte.

Gevolgtrekkings: Ten spyte van beperkings, het hierdie studie die voorspellende waarde van KP in risiko van siekte-terugval bevestig. 'n Meer pasgemaakte benadering tot behandeling is moontlik deur gebruik van hierdie merker. Die verband tussen intermediêre monosietuitbreiding, KP en siektefenotipe moet ook verder ondersoek word. Toekomstige studies wat behandelingsnaïewe deelnemers insluit, sal voordelig wees om die bruikbaarheid van KP in die monitering van reaksie op behandeling te bepaal.

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List of Abbreviations

.fcs	Flow cytometry standard file
ACPA	Anti-citrullinated protein antibodies
ANA	Antinuclear antibody
APC	Allophycocyanin
BB	Brilliant blue
BV	Brilliant violet
CCL	CC Chemokine ligand
CCP	Cyclic-citrullinated peptide
CD	Cluster of differentiation
COVID	Coronavirus disease
CRP	C-reactive protein
CS&T	Cytometer setup & tracking
CXCL	CXC Chemokine ligand
DAMPS	Damage-associated molecular patterns
DMARDS	Disease-modifying antirheumatic drugs
ELISA	Enzyme-linked immunosorbent assay
ERA	Enthesitis-related arthritis
ESR	Erythrocyte sedimentation rate
FA	Folic acid
FACS	Fluorescence activated cell sorting
FBC	Full blood count
FMO	Fluorescence minus one
FSC	Forward scatter
GPS	Guided panel solution
HCQ	Hydroxychloroquine
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HREC	Health Research Ethics Committee
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
ILAR	International league of associations for rheumatology
INH	Isoniazid
IP-10	IFN-gamma-inducible protein 10
JADAS	Juvenile arthritis disease activity score
JIA	Juvenile idiopathic arthritis
MAS	Macrophage activation syndrome
MCP	Monocyte chemoattractant protein
MFI	Median fluorescence intensity
MIF	Macrophage migration inhibitory factor
MRP	Myeloid-related protein
MTX	Methotrexate
NHLS	National Health Laboratory Service
NSAIDS	Non-steroidal anti-inflammatory drugs
OD	Optical density
Oligo-JIA	Oligoarticular juvenile idiopathic arthritis
PAMPS	Pathogen-associated molecular patterns
PE	Phycoerythrin
PenVK	Phenoxymethylpenicillin
PGALS	Paediatric gait, arms, legs, and spine
Poly-JIA	Polyarticular juvenile idiopathic arthritis
PRR	Pattern recognition receptors
PsA	Psoriatic juvenile idiopathic arthritis
RA	Rheumatoid arthritis
RCF	Relative centrifugal force
RF	Rheumatoid factor
sJIA	Systemic juvenile idiopathic arthritis
SSC	Side scatter
TB	Tuberculosis
TBH	Tygerberg hospital
Th	T Helper
TLR	Toll-like receptor
TMJ	Temporomandibular joints

TNF	Tumour necrosis factor
uJIA	Undifferentiated juvenile idiopathic arthritis

Chapter 1 Introduction

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease affecting juveniles aged 16 years or younger. Characteristic features of the disease include persistent arthritis which continues for at least 6 weeks and is not a result of any other inflammatory condition. The cause of JIA is unknown however it is likely due to a combination of genetic and environmental factors that enhance a person's susceptibility (Al-Mayouf et al. 2021). In South Africa, and especially the areas surrounding (TBH) in the greater Cape Town metropole, the lower socio-economic status and reliance on the state-funded health system delays patient access to care and inevitably prolongs the time taken to achieve remission. There is also a lack of paediatric rheumatologists and medical expertise surrounding this disease in this setting (Weakley et al. 2012).

Currently, the routine monitoring of disease activity of JIA patients is based on diagnostic laboratory evaluation of inflammation based on C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). However, these tests only detect systemic inflammation and tend to correlate poorly with disease activity (Foell and Däbritz 2011). In contrast, levels of plasma calprotectin, a member of the S100 protein family, have been more strongly associated with disease activity as the marker is indicative of local inflammation and is released by activated monocytes and macrophages at the affected joints (Stříž and Trebichavský 2003; García-Arias et al. 2013). Gerss et al. have shown the potential of calprotectin to detect sub-clinical inflammation and subsequent risk of relapse in JIA (Gerss et al. 2012). Despite the general usefulness of standard inflammatory markers, there is a need for a more disease- and lesion-specific marker, like calprotectin, in predicting the course of JIA. This marker could allow for customisation of treatment plans in order to achieve and maintain disease remission more effectively (Inciarte-Mundo et al. 2018).

In this study, plasma concentrations of calprotectin (S100A8/A9) and multiple inflammatory biomarkers were assessed to in order to delineate trends in inflammatory profiles relating to disease activity and routine diagnostic laboratory markers over time. In addition, how S100A8/A9 compares with other markers was evaluated.

The goal of treatment and monitoring is to maintain remission and improve the child's quality of life. Accurate detection of sub-clinical inflammation, and access to a predictive marker of disease activity, could effectively shorten the time between flare or active disease and disease remission to achieve this goal.

Chapter 2 Literature Review

2.1. Sub-Saharan Africa and JIA

Sub-Saharan Africa is home to an estimated 390 million children (Scott and Webb 2014). JIA, a rare autoimmune disease, is affecting approximately 3.8 to 400/100 000 children according to worldwide prevalence studies (Al-Mayouf et al. 2021). Accurate statistics for South Africa do not exist and additional studies are necessary to observe JIA prevalence in this region (Weakley et al. 2012; Usenbo et al. 2015). This autoimmune disease, although perceived to be rare, is no less common that other non-communicable diseases affecting children in South Africa, such as, diabetes which affects roughly 0.8/100 000 and epilepsy affecting approximately 17.4/100 000 children per year (Scott and Brice 2015; Kalweit et al. 2015; Wagner et al. 2015). The observed low prevalence of JIA cases, compared to that of more common non-communicable diseases, can be attributed to delayed referral at primary health care facilities and education surrounding the disease in this region (Weakley et al. 2012). JIA shares many clinical and physiological characteristics with adult rheumatoid arthritis (RA) (Prahalad and Glass 2002). Global prevalence of JIA and RA are paralleled, with JIA affecting roughly 3.8-400 per 100 000 children and adult RA which affected roughly 460 per 100 000 adults between 1980 and 2019 according to a recent meta-analysis study (Almutairi et al. 2020; Al- Mayouf et al. 2021).

There is competition for resources in a setting such as Sub-Saharan Africa, which is facing both social and infectious disease burdens. This competition results in the focus being directed at communicable diseases such as the human immunodeficiency virus (HIV) pandemic and tuberculosis (TB), which are major health problems facing this region. Therefore, there is an apparent scarcity of resources in the paediatric rheumatology discipline. This is evident due to a shortage of trained specialists, support services and education surrounding juvenile rheumatic diseases (Scott and Webb 2014). Noticeably, the recent Covid-19 pandemic has also demonstrated how health resources can effectively be diverted from what are perceived as rarer, less threatening diseases such as JIA.

Very few studies have been performed in South Africa to describe the prevalence of JIA in communities and across provinces. A recent study however, evaluated the disease at two tertiary institutions in the Western Cape, South Africa. These institutions included the Groote Schuur Hospital and TBH which are affiliated with the University of Cape Town and Stellenbosch University, respectively. The study compared its findings with that of countries such as India, Europe, and America to observe the variation in prevalence of disease subtypes (discussed below) among different populations. One limitation of this study was the small sample size in the South African

cohort compared to those of the other regions which highlights the possibility of an underestimate due to poor access to healthcare in this setting (Weakley et al. 2012).

2.2. JIA

The international league of associations for rheumatology (ILAR) definition for JIA has been widely accepted and defines JIA as arthritis, occurring before or at the age of 16, which persists for at least 6 weeks and is exclusive of any other medical condition, most specifically, that of an inflammatory nature (Weakley et al. 2012). JIA is recognised as an autoimmune disease due to the involvement of T and B cells which form part of the adaptive immune response. Autoimmune and auto-inflammatory diseases differ in their immunological involvement. While autoimmune pathology predominantly involves the adaptive immune response, auto-inflammatory diseases involve the innate immune response, which comprises cytokines and inflammatory mediators (Liang et al. 2017). Given the involvement of inflammatory markers in disease progression of JIA, it may be better described as an autoimmune disease characterised by inflammatory episodes involving both the innate and adaptive immune responses.

The term JIA is an all-encompassing term for several subtypes of childhood arthritis previously considered as separate conditions or classified collectively under redundant names such as juvenile rheumatic arthritis or juvenile chronic arthritis (Barut et al. 2017). The ILAR categorises JIA into 7 subtypes according to presentation of similar disease characteristics and laboratory results. These mutually exclusive subtypes consist of: oligoarthritis, polyarthritis rheumatoid factor (RF)-negative, polyarthritis RF-positive, systemic juvenile idiopathic arthritis (sJIA), enthesitis-related arthritis (ERA), psoriatic arthritis, and a final subtype undifferentiated juvenile idiopathic arthritis (uJIA), consisting of cases which fit none or more than one of the first six subtypes (Martini et al. 2019). While there are differing subtypes, the feature that all subtypes share is inflammation of one or more joints, resulting in arthritis. South African and Indian studies recognise polyarticular JIA as being more common than oligoarticular JIA in the population while the opposite finding was evident in European and American based studies (Scott and Brice 2015). Varying prevalence of the disease worldwide has proven problematic in isolating a single genetic or environmental factor responsible for disease pathology (Barut et al. 2017).

2.2.1. Aetiopathogenesis

The research surrounding JIA has defined the cause of JIA as an ‘unclear’ or ‘poorly understood’ process. However, there are a few factors which have been identified as contributors to its pathogenesis: Genetic predispositions, environmental contributors, and the immune system, all of which are linked to an individual’s susceptibility. It is theorised that the complex interactions between these factors result in immune disruptions which are responsible for this autoimmune disease. An

exception can be made in the case of sJIA: while JIA is generally seen as autoimmune, this subtype appears predominantly auto inflammatory in nature due to an over-activation of the innate immune response driven by inflammatory mediators. JIA is considered autoimmune due to the involvement of the T and B cells which are responsible for detectable autoantibodies. Autoantibodies associated with the subtypes of JIA include anti-nuclear antibodies (ANAs), RF and anti-citrullinated protein antibodies (ACPA), amongst others. Irrespective of the terminology assigned to each subtype, inflammatory cytokines are involved in the manifestation of clinical signs and symptoms resulting from disease. Research focusing on gut microbiota, which is thought to play a pivotal role in autoimmune diseases, is now emerging in relation to JIA which may add to current theories of causality (Ravelli and Martini 2007; Hahn and Kim 2010; Scott and Brice 2015; Barut et al. 2017; Mahmud and Bindstadt 2019).

Genetic predispositions have been studied and the human leukocyte antigens (HLA), such as HLA-B27 and HLA-DRB1, and non-HLA genes, such as PTPN22 and IL2RA, have regularly been implicated in cases of JIA. Environmental contributors may include both past infections and the composition of the gut microbiome. Some infections currently under investigation for its role in JIA pathogenesis include infections occurring in the gut, common childhood viruses (rubella, mumps, parvovirus B19, Epstein-Barr virus) and mycoplasma, however, this list is not exhaustive (Barut et al. 2017; Nigrovic et al. 2019).

Inflammation and joint damage

A joint is a feature existing between two bones and consists of a synovial fluid filled space enclosed by a synovial membrane and in immediate contact with the cartilage on the surface of the bone. The damage to the joint is due to inflammation of the synovial membrane which occurs when inflammatory cells permeate the synovial fluid and release degradation enzymes. Joint inflammation is the defining feature of juvenile arthritis and is commonly seen across all subtypes of JIA (Muller et al. 2015).

Figure 2.1 illustrates a cross-sectional view into the knee joint of a healthy child (a), and a JIA affected child (b). A child suffering with an affected joint may experience an increase in synovial fluid surrounding the bones, a thinning of cartilage and inflammation of the synovial membrane (Chang 2010).

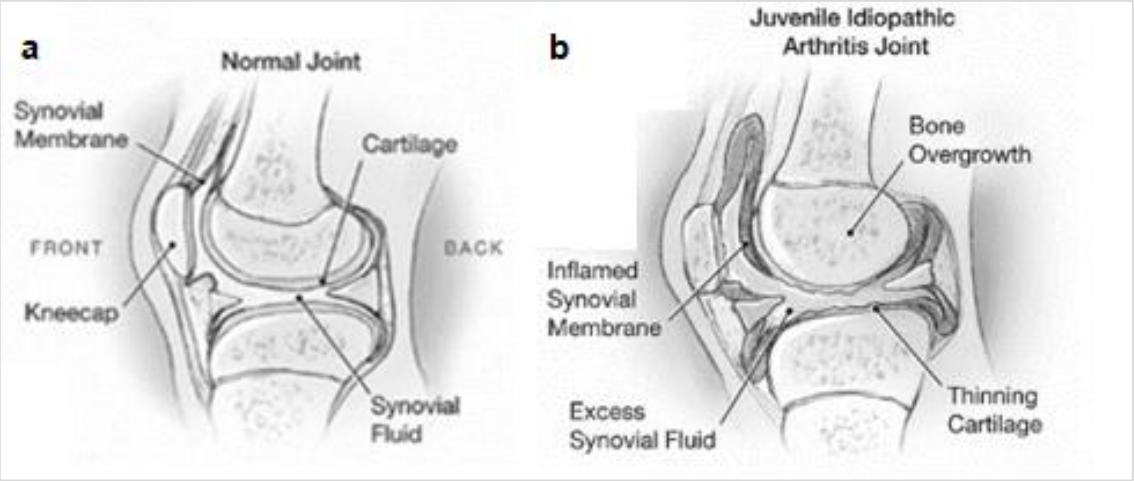


Figure 2.1. Cross-sectional illustration of a healthy knee joint (a) and the affected knee joint (b) of a child with JIA showing key features of bone overgrowth, inflamed synovium, excess synovial fluid and thinning cartilage (Chang 2010).

Inflammation and the immune response go hand-in-hand when evaluating the physical evidence of JIA. Figure 2.2 schematically details the process by which joint, and bone damage occur in cases of JIA which lead to an arthritic phenotype. A subset of T lymphocytes (T helper 1 cells) is at the centre of this interacting network of cells and cytokines in the synovial fluid of the affected joint. The synovial fluid also contains B lymphocytes, macrophages, monocytes, osteoclasts, and synovial fibroblasts, among others. The CD4+ T helper 1 (Th1) cells are a subset of cytokine producing cells which release interferon-γ (IFN-γ), a key initiator of inflammation. These Th1 cells promote the release of inflammatory mediators by monocytes, macrophages, and synovial fibroblasts. Additionally, they stimulate B cells to release immunoglobulins such as ANAs and RF (Hahn and Kim 2010).

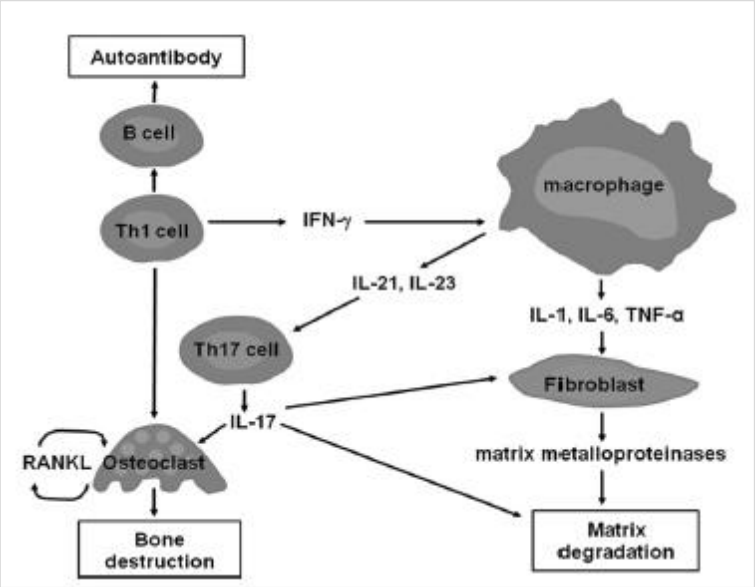


Figure 2.2. Bone destruction, matrix degradation and joint damage resulting from interactions of Th1 cells with B cells, macrophages, monocytes, fibroblasts, and osteoblasts aided by a network of cytokines (Hahn and Kim 2010)

Several cytokines are released by activated monocytes and macrophages following stimulation by IFN-γ. These include interleukin (IL)-1α and IL-1β, IL-6 and tumour necrosis factor (TNF)-α. The action of these cytokines on the synovial fibroblasts results in the production of matrix metalloproteases which degrade cartilage. Further expression of IL-21 and IL-23, by activated monocytes and macrophages, induce production of IL-17 by another subset of T lymphocytes, namely T helper 17 (Th17) cells. The action of this proinflammatory cytokine encourages the bone absorbing properties of osteoclasts and supports the cartilage destruction pathway via the synovial fibroblasts. Furthermore, the action of IL-1 and TNF-α hinders the ability of fibroblasts to produce metalloprotease inhibitors. The combined interactions of cytokines and synovial cells at the joint results in the cardinal signs of inflammation such as heat, swelling, pain, redness, and loss of function (Hahn and Kim 2010). The overall process of cartilage and bone destruction have the potential to result in growth retardation and functional disabilities (Muller et al. 2015).

2.2.2. Subtypes of JIA

There are several subtypes of JIA, each with their own unique disease profile. Several characteristics for each subtype exist and these aid in assigning a diagnosis, together with clinical results and a thorough patient history (Scott and Brice 2015). A short description and list of pathophysiological characteristics for each subtype are described and summarised in Table 2.1 below:

Oligoarticular JIA (oligo-JIA) is more commonly seen in female children under the age of 6 years. It is the most observed subtype in developed countries such as America and Europe. Oligoarticular JIA cases can be further divided into two distinct subgroups: persistent and extended oligo-JIA.

Persistent oligoarticular JIA involves between 1 and 4 affected joints while extended oligo-JIA involves more than 4 affected joints occurring within the first 6 months of the disease.

The lower limb joints, for example the knees and ankles, are most affected in this subtype. However, additional upper limb joints may be involved in extended oligo-JIA cases. Clinical presentations of oligo-JIA include swelling of these joints (usually warm to the touch), together with some morning stiffness of joints while pain typically remains absent. Routinely monitored laboratory markers for this subtype include ANAs, ESR and CRP. Roughly 70–80% of cases will test positive for ANAs which may be accompanied by uveitis, a common symptom of juvenile arthritis. The CRP and ESR are inflammatory markers which may be elevated before proceeding with anti-inflammatory treatment. The prognosis however remains encouraging in cases of oligoarticular JIA as there is usually no functional disabilities or delayed growth. A small period of follow-up, usually up to 5 years, is recommended upon achieving remission as chances of a disease flare up is possible (Barut et al. 2017; Scott and Brice 2015).

Polyarticular JIA (poly-JIA), similar to oligo-JIA, is more frequently seen in females and consists of two distinct subgroups: RF-positive and RF-negative, also referred to as seropositive and seronegative poly-JIA respectively. Although prevalence data is subjective to region, seronegative cases are usually seen more than seropositive (Weakley et al. 2012). In addition, seropositive is more likely to develop in adolescents while seronegative poly-JIA is more common in younger children (Scott and Brice 2015).

Patients from both subgroups may be anaemic and experience fever and weight loss. Additionally, delays in growth may result due to disease pathology. Poly-JIA involves 5 or more joints during the initial 6 months of the disease and can often present as oligo-JIA, delaying the poly-JIA diagnosis until the disease progresses. Seronegative poly-JIA can be further divided into 3 subgroups based on joint symmetry, clinical manifestations, and associated laboratory markers: The first is associated with asymmetrical joint involvement, greater risk of uveitis and positive laboratory testing for ANAs and a genetic marker, HLA-DRB1, which is associated with increased risk of arthritis development. This first subgroup is sometimes disguised as oligo-JIA given the similar clinical presentation; The second presents with symmetrical joint involvement, unpredictable clinical manifestations, and a marked increase in ESR, a marker of bodily inflammation. It is also comparable to seronegative adult RA; The third subgroup, also referred to as dry synovitis, is arguably the worst subgroup as it tends to respond poorly to treatment strategies and subsequently results in a poor prognosis. It is a poorly understood subgroup and laboratory definitions remain unclear (Ravelli and Martini 2007; Barut et al. 2017).

Seropositive poly-JIA, not unlike seronegative poly-JIA, involves both small and large joints of the upper and lower limbs. Joint involvement is typically symmetrical as seen in Figure 2.3. As

mentioned, it involves 5 or more joints and mostly affects adolescent females. RF will test positive on two separate instances, at least 3 months apart in these cases. Accompanying clinical presentations may include rheumatoid nodules in about a third of confirmed cases during the initial year of disease onset. This subgroup is also considered the equivalent of seropositive adult RA (Ravelli and Martini 2007).

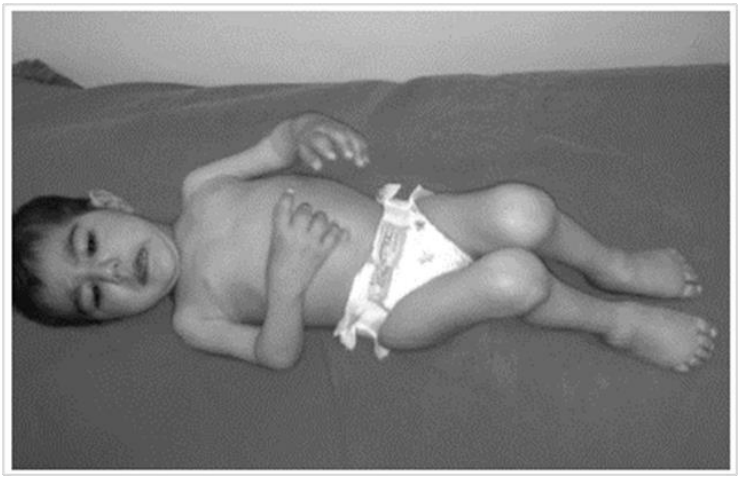


Figure 2.3. Symmetrical small and large joint involvement in polyarticular JIA (Barut et al. 2007)

The presenting arthritis in poly-JIA patients predominantly involve small joints in a symmetrical fashion, however, large joints may also be involved in conjunction with the smaller joints. The small joints of the upper and lower limbs include those in the wrists, hands, and feet. In contrast, the large joints include the knees, hips, and ankles. The temporomandibular joints (TMJ), along with the cervical spine may also be affected in patients with poly-JIA. There may be some serious consequences of continued joint inflammation in poly-JIA patients and may inevitably demand surgical interventions – this is especially seen where hip joints are involved (Weiss and Ilowite 2005; Barut et al. 2017).

ERA is one of the most complex subtypes of JIA due to heterogeneity among cases. This subtype is more often seen in males over the age of 7. Most prevalence studies done worldwide have identified ERA as the least common subtype of JIA, with the exception of some countries such as Taiwan, India and South Africa (Weakley et al. 2012; Shih et al. 2019).

Enthesitis refers to inflammation of the entheses (the sites at which the tendons attach to the bone) and is one of the main clinical characteristics of the disease. Enthesitis is also characterised by pain and tenderness. The most common tendon affected is the Achilles (Weiss and Ilowite 2005; Barut et al. 2017). Additional characteristics of the disease may also include negative testing for RF and ANAs, positive testing for HLA-B27, a family history of HLA-B27-related diseases, inflammatory back pain, acute uveitis and arthritis of joints along the axial skeleton and lower body (Weiss 2012; Shih et al. 2019). Joint involvement is likely to be asymmetrical, affecting the lower body. The knees,

ankles and sacroiliacs appear to be affected in most cases. While ERA can initially appear to be oligo-JIA, due to similarly affected joints, the main distinguishing factor is the involvement of the hip joint (sacroiliitis). In contrast to other subtypes, ERA appears to have the worst prognosis, especially if cases go untreated. Literature suggests untreated ERA in childhood causes disease escalation which results in adult ankylosing spondylitis. Early recognition and treatment of any JIA subtype is therefore paramount in order to prevent physical consequences later in life (Barut et al. 2017).

Psoriatic JIA (PsA), like ERA, is another complex subtype of JIA. It is characterised by arthritis together with a psoriatic rash. In cases where the rash is absent, the arthritis may be accompanied by two of the following extra-articular factors: dactylitis - inflammation and swelling of finger joints as seen in Figure 2.4, nail changes and evidence of psoriasis in a first-degree relative. Diagnosis of this subtype can prove difficult due to the articular arthritis occurring long before the extra-articular symptoms (Ravelli and Martini 2007; Barut et al. 2017).

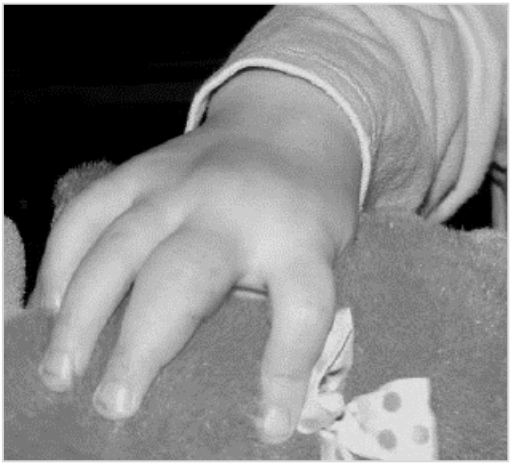


Figure 2.4. Dactylitis in a young patient diagnosed with psoriatic arthritis (Ravelli and Martini 2007)

The joints involved in this subtype are variable, affecting both small and large joints, symmetrically and asymmetrically. Testing for ANAs is mostly positive while HLA-B27 is only found in some cases. Symptoms of ERA, for example enthesitis and sacroiliitis, can also be seen in these cases. The variety of factors attributing to the diagnosis makes PsA difficult to define by an organised set of characteristics. The subtype is therefore a controversial topic of discussion in the field of paediatric rheumatology (Barut et al. 2017).

sJIA is a non-discriminatory subtype because it affects both female and male children with equal frequency and can occur at any age. A diagnosis of sJIA is made if the patient has arthritis together with fever for at least two weeks, as well as one of the following accompanying symptoms: rash, hepatosplenomegaly, generalised lymphadenopathy or serositis. Several factors are considered when making a differential diagnosis of sJIA considering it shares symptoms with diseases such as Kawasaki disease and other autoinflammatory diseases. The factors to be considered included

bacterial and viral infections, cancerous growths and other rheumatic diseases which may cause similar symptoms (Ravelli and Martini 2007, Barut et al. 2017).

The arthritis is usually symmetrical and polyarticular, involving both the small and large joints. Arthritis may be present at onset or develop as the disease progresses. The abovementioned fever is intermittent and can peak twice daily reaching a temperature of 39.5°C. Figure 2.5 shows the rash which often occurs in conjunction with the fever (Barut et al. 2017).

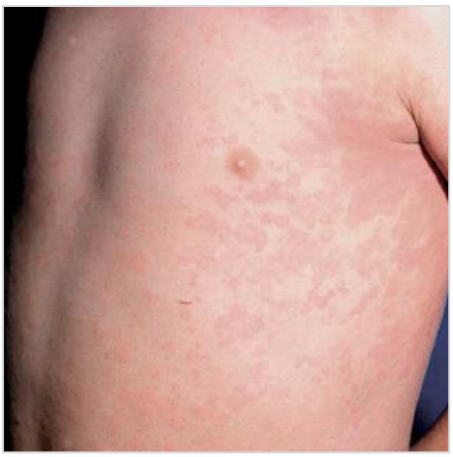


Figure 2.5. Common salmon-pink rash of sJIA (Ravelli and Martini 2007)

There are rare cases in which macrophage activation syndrome (MAS) complicates the prognosis. Development of MAS is life-threatening and is associated with persistent fever, hepatosplenomegaly, neurological and haemorrhagic symptoms. In addition to the clinical manifestations of sJIA, laboratory testing may show elevated levels of ferritin, ESR and CRP, as well as thrombocytosis.

sJIA can result in more serious long-term problems such as osteopenia, amyloidosis, and stunted growth. However, early detection and intervention is key in preventing long term complications (Ravelli and Martini 2007; Barut et al. 2017).

uJIA is representative of cases which fit into none or more than one of the abovementioned subtypes. Prevalence studies have shown that very few cases are diagnosed as undifferentiated (Weakley et al. 2012).

A comparative review of JIA subtypes among different populations worldwide was included in a South African study in 2012. The study compared the prevalence data of each JIA subtype across South Africa, Morocco, Western and Eastern Europe, India, Turkey, and Latin America. The relevant data was all obtained through tertiary institutions involving registered paediatric rheumatologists.

Data for psoriatic, ERA and uJIA were only available from South Africa, India, and Turkey. Psoriatic and uJIA were the least frequent subtypes across these regions with as little as 1-5% of their

respective JIA cases belonging to these subtypes. The review confirms polyarticular JIA as being the most prevalent in South Africa (40.9%) while oligoarticular JIA appears to be the dominant subtype in Western Europe (51%). The review further identifies sJIA mostly affecting regions such as Morocco (26%) and Latin America (28.5%) and accounting for less than 10% of cases in India and South Africa. The comparison highlights the diverse prevalence of subtypes across different regions of the world (Weakly et al. 2012).

Table 2.1: Summarised ILAR characteristics for each JIA subtype (Adapted from Mahmud and Binstadt 2019; Scott and Brice 2015)

JIA Subtype	Characteristics
OligoJIA	Younger child More common in females Involvement of 1 – 4 joints within first 6 months Persistent vs. Extended (additional joints) ANA positive in roughly 70% of cases Uveitis common
(RF+ polyJIA)	Adolescents More common in females Involvement of ≥ 5 joints within first 6 months Presence of RF
(RF- polyJIA)	Younger child More common in females Involvement of ≥ 5 joints within first 6 months Absence of RF ANA positive in roughly 40% of cases
ERA	Older child More common in males Enthesitis Sacroiliitis and inflammatory back pain HLA-B27 positive Family history of HLA-B27 related disease
PsA	Psoriasis Dactylitis Nail changes Evidence of psoriasis in 1st-degree relative
sJIA	Persistent fevers Rash Hepatosplenomegaly Serositis Complicated by MAS
uJIA	Chronic idiopathic arthritis which fits more than one or none of the categories

ERA, enthesitis-related arthritis; ANA, antinuclear antibody; RF, rheumatoid factor; MAS, macrophage activation syndrome

2.3. Diagnosis of JIA

A diagnosis of JIA can prove challenging due to the nature of juvenile development and growth. In addition, many juveniles and/or parents do not report any joint pain or mobility issues. Therefore, identifying this disease depends on a combination of clinical assessment, history, and the experience of the medical professional. In order to reach a diagnosis, a thorough examination must be done to exclude all other causes of arthritis or musculoskeletal pain. The physical exam will also identify the number of active joints. The current screening tool used in the Western Cape is the paediatric gait arms legs spine (PGALS). This tool consists of three easy questions and several planned movements which can easily identify stiff or painful joints which will then be examined further. Following this, serological investigations are done in order to identify disease complications, alter treatment strategies and exclude other causes of joint pain. Current serological tests include a full blood count (FBC), screening for HLA-B27 and quantification of CRP, ESR, ANA, RF and anti-cyclic citrullinated peptides (anti-CCPs). A thorough patient history is also important in identifying a case of JIA, this may include onset and duration of joint pain, exposure to infectious diseases and a family history (Scott & Brice, 2015). Additionally, a juvenile arthritis disease activity score (JADAS) is assigned which scores disease activity based on information provided by parents, active joints, and levels of acute phase proteins such as CRP and ESR. This score can be used as a monitoring tool throughout treatment in order to monitor changes in disease activity (Consolaro et al. 2016).

Following diagnosis, regular measurement of disease activity is essential for monitoring changes in disease progression over time and importantly, response to treatment. Disease activity relies on a number of variables which have been previously established by the American College of Rheumatology. These variables include physician assessment, patient/parent's assessment of the patient's wellbeing, the number of active joints, and laboratory measures of inflammation. The above criteria are used at the paediatric rheumatology clinic at TBH to score disease activity and are collectively referred to as the JADAS. This compound score allows the physician to classify disease activity into the following categories: inactive, low, moderate, or high disease activity as indicated in participant files (Consolaro et al. 2009; Consolaro et al. 2016).

2.4. Treatment of JIA

Disease remission is the main goal of treatment in cases of JIA. Complete remission prevents complications of the disease such as physical disability, pain, uveitis, stunted growth and in some cases, MAS. Clinically inactive disease or remission can be defined by cut-off values of JADAS. The frequency at which patients reach remission is ever growing due to modern medications (Scott and Brice 2015; Shoop-Worrall et al. 2017).

Non-steroidal anti-inflammatory drugs (NSAIDs)

The main action of NSAIDs include pain relief and the reduction of inflammation. In most cases, NSAIDs are administered as first line therapies and the effects of these drugs are relatively fast (1 – 3 days). They are generally well tolerated in juveniles; however, some side effects may be experienced such as abdominal pains and headaches (Scott and Brice 2015; Barut et al. 2017; Ravelli and Martini 2007).

Corticosteroids

The effects of corticosteroids have far greater anti-inflammatory activity when compared to NSAIDs. However, the possible toxic side effects of corticosteroid administration make it an unnecessary course of treatment in cases where NSAIDs can control symptoms. It is therefore often reserved for extreme cases of poly-JIA and sJIA. Induction of remission has been seen in cases of oligo-JIA when administering intra-articular injections. Furthermore, oral, or intra-articular administration of corticosteroids has been implicated in the reduction of swelling and persistent fever in cases of sJIA (Scott and Brice 2015; Barut et al. 2017).

Disease modifying anti-rheumatic drugs (DMARDs)

DMARDs are considered safe and effective and are valuable in reducing the need for NSAIDs and steroids which have adverse side effects. An improved prognosis relies on timely administration of DMARDs; this has also been linked to a decrease in disease severity (Scott and Brice 2015).

One commonly used DMARD is methotrexate (MTX). It is considered non-toxic and has proven greatly successful for the management of persistent arthritis. Treatment is administered weekly, and a response can be seen within one to two weeks. In order to manage side effects such as nausea and vomiting, folic acid is taken daily to control the potency of MTX (Scott and Brice 2015; Barut et al. 2017).

Biologic agents

Advancements in developing biologic agents has revolutionised the treatment of JIA and have made remission a likely outcome in many cases. A major drawback of this treatment, however, is accessibility due to the high costs involved in developing these individualised therapies. In contrast to the expensive nature of this treatment option, the benefits of using biologic agents increases the chances of disease remission and has also been effective in decreasing disease severity. This treatment option is also ideal in cases where conventional antirheumatic therapy is ineffective. Additionally, the safety of using biologic agents for treating JIA has been proven. Some major classes of biologic treatments used in JIA include TNF inhibitors, IL inhibitors and B-cell inhibitors (Scott and Brice 2015; Barut et al. 2017; Ravelli and Martini 2007).

In addition to medical treatment, physical therapy is beneficial for joint mobility and pain management. Furthermore, the effect of chronic pain and limited mobility on the mental well-being of both patients and family can be difficult. In order to ensure the psychosocial health of patients, in addition to physical health, affected families may benefit from the help of mental health workers (Scott and Brice 2015).

2.4.1. Subclinical Inflammation and Risk of Relapse

Subclinical inflammation is a concern when assessing disease activity and is responsible for relapse in patients thought to be in stable remission. Subclinical disease activity cannot be monitored using standard laboratory assessments and makes it difficult to determine when a patient is ready to be weaned off treatment. The presence of subclinical disease activity highlights the need for a biomarker which can be used to determine the chance of relapse in otherwise stable patients (Duurland and Wedderburn 2014). Calprotectin has been investigated in relation to risk of relapse in patients with apparent inactive disease activity. While the standard markers, CRP and ESR, cannot predict the future course of the disease, plasma calprotectin reflects ongoing inflammation of the synovium, including otherwise undetectable subclinical inflammation. Studies have confirmed its use as a predictive marker for the risk of disease relapse (Gerss et al. 2012). Disease flares/relapse have also been shown to be less frequent in a disease remission cohort continuing treatment than a remission cohort discontinuing treatment (Foell et al. 2010). There have not however been extensive studies comparing calprotectin to a range of other biomarkers of interest, standard diagnostic markers, and monocyte changes in the same cohort.

2.5. Monocytes

Monocytes, and their subset distribution, are immunologically significant in many inflammatory diseases and have been implicated in both stimulating and resolving inflammation. Whole blood flow cytometry is the ideal method for investigating monocyte subset distribution because it reduces the risk of unintended cellular activation by reducing the handling of samples (Marimuthu et al. 2018). Due to the importance of monocytes and macrophages in inflammation, including joint inflammation, these cells require more detailed study in this disease setting.

The immune system relies heavily on the action of monocytes as it has roles in both the innate and adaptive immune responses. These white blood cells are partly responsible for regulating inflammation in the body and have therefore been implicated in many inflammatory diseases. Monocytes are typically divided into three distinct subsets: Classical, intermediate, and non-classical. These account for ~85%, ~5% and ~10% of all monocytes, respectively. The altered distribution of these subsets may have pathological implications. The expression of surface markers, such as cluster of differentiation (CD)14 and CD16, are what makes these subsets phenotypically

different. The expression of these surface markers for each subset is shown in Figure 2.6; Classical monocytes express CD14 highly (CD14++CD16-), intermediate monocytes express moderate levels of CD16 in addition (CD14+CD16++/CD14++CD16+) and finally non-classical monocytes express low levels of CD14 with higher CD16 expression (CD14+CD16++). The intermediate subset accounts for a low percentage of circulating monocytes, however, the subset expands in cases of inflammation. This has led to the identification of intermediate monocytes as key role players in inflammatory diseases (Ziegler-Heitbrock et al. 2010; Marimuthu et al. 2018).

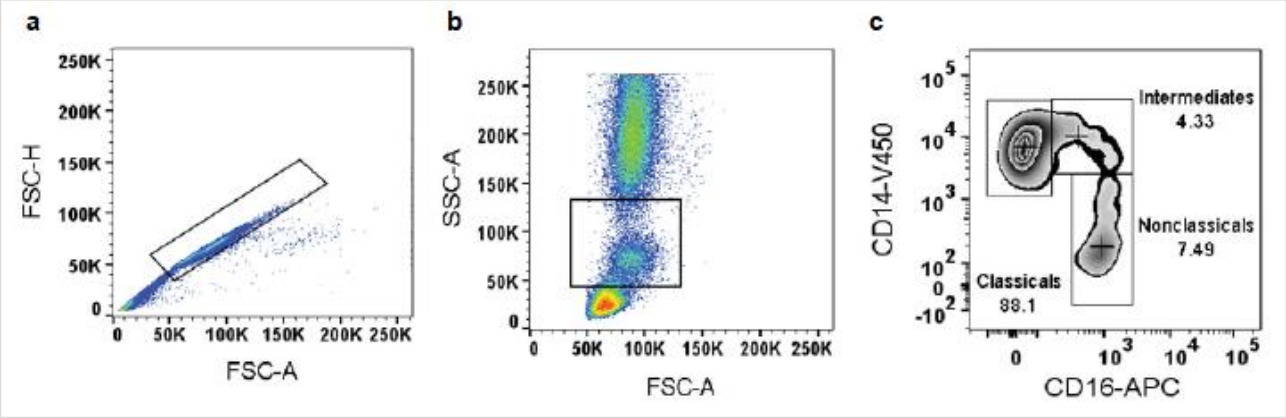


Figure 2.6: Cytometric analysis of whole blood monocytes. Gating of singlets to remove any cell clusters and debris (a), gating on monocyte population (b) and further sorting of monocyte subsets based on expression levels of CD14 and CD16 (c) surface markers (Marimuthu et al. 2018).

FSC-H, forward scatter height; FSC-A, forward scatter area; SSC-A, side scatter area; APC, allophycocyanin; V450, violet laser reagent.

In addition to CD14 and CD16, monocytes can be further characterised by expression of CD64; The expression of this molecule can be upregulated in response to tissue damage. Like CD64, CD36 is also highly expressed on mature monocytes, and it acts as a collagen or thrombospondin ligand (Lambert et al. 2017). The co-stimulatory molecule, CD86, expressed on monocytes is important for interactions with lymphocytes in order to prime the adaptive immune response (Pinto et al. 2018).

Furthermore, surface expression of the scavenger receptor, CD163, has also been identified in monocyte and macrophage subsets. The secretion of soluble CD163 by monocytes can occur following ligand binding to surface receptors, such as toll-like receptor (TLR)-4, as a result of inflammation. Soluble CD163 has also been implicated in anti-inflammatory processes linked to autoimmune diseases (Tippett et al. 2011).

There are a few methods which can be used to assess cellular populations in a whole blood sample, including monocyte populations. One such approach is the use of fluorescence-activated cell sorting (FACS) which is a specialised form of flow cytometry. This method allows for sorting of the cells in a sample by staining with several fluorescence-labelled antibodies in order to identify target populations (Julla et al. 2019).

There have been several studies examining monocytes in the context of JIA and other similar inflammatory diseases. For example, Prokopec et al. found that monocyte numbers are significantly increased in JIA patients when compared to a healthy, age and sex matched control group. The study consisted of 26 confirmed JIA cases and 21 healthy controls. Peripheral blood was collected from all participants and peripheral blood mononuclear cells were isolated for flow cytometry analysis. Each sample of cells was sorted according to size and granularity in order to isolate the monocyte population. The percentage of these monocytes in the JIA groups as compared to the healthy control group showed a significant increase. The data confirms the involvement of monocytes in JIA pathogenesis and its potential role in the production and release of pro-inflammatory cytokines (Prokopec et al. 2012).

Additionally, Gaur et al. found that peripheral blood monocyte frequency in ERA patients increased significantly ($p < 0.005$) when compared to healthy controls across all monocytes. The study consisted of 46 ERA patients, 17 disease controls (i.e., other JIA subtypes) and 17 healthy controls. Peripheral blood was collected from all participants and used for flow cytometry analysis to calculate subset frequency (Gaur et al. 2016).

2.6. Biomarkers

Biomarkers are measurable components of patient samples generally in the form of saliva, blood, or urine. When selecting biomarkers of interest in relation to disease monitoring or diagnosis, they should hold stable enough within the sample to be measured using reproducible assays. The potential for JIA specific biomarkers exists and would allow for personalised treatment strategies which in turn could shorten the time taken to achieve disease remission (Duurland et al. 2014).

Inflammation of the synovial membrane (synovitis) characterises an arthritic joint. The inflammation may be physically evident in the form of swelling, restricted motion, heat, and a red skin tone at the affected area. Pro-inflammatory cytokines are key players of the inflammatory process, and this has been confirmed following the effectiveness of anti-inflammatory drugs, however, this treatment is not always effective and may need to be supplemented with individualised therapy or biologics. The concentration of cytokines and chemokines can be measured in a patient plasma sample given that circulating cytokines are representative of local inflammation (de Jager et al. 2007; Hahn and Kim 2010). There are also a number of standard tests and abnormalities which are evaluated and are indicative of systemic inflammation. These include assessments of platelet counts, transaminases, ESR and CRP, as well as more specialised markers such as ferritin, aldolase, d-dimers and procalcitonin. The availability of these more specialised tests is, however, limited in a setting like South Africa given the limitations on healthcare resources (Weakly et al. 2012; Trachtman et al. 2021; Zaripova et al. 2021). Additionally, members of the calgranulin family, such as the S100A8/A9 and S100A12 proteins, have been investigated as more sensitive markers of inflammation in various

inflammatory disorders, including arthritis (Vaos et al. 2013; García-Arias et al. 2013; Zaripova et al. 2021).

Figure 2.7 displays the process of biomarker production in response to synovitis. Cytokines are released early in response to tissue damage but are unstable. In contrast, danger-associated molecular patterns (DAMPS) such as calprotectin are simultaneously released and are stable indicators of local tissue damage. Lastly, acute phase proteins, produced by the liver in response to circulating inflammatory cytokines, are also indicators of inflammation, however, these may indicate systematic inflammation as opposed to inflammation as a result of synovitis (Foell and Däbritz 2011).

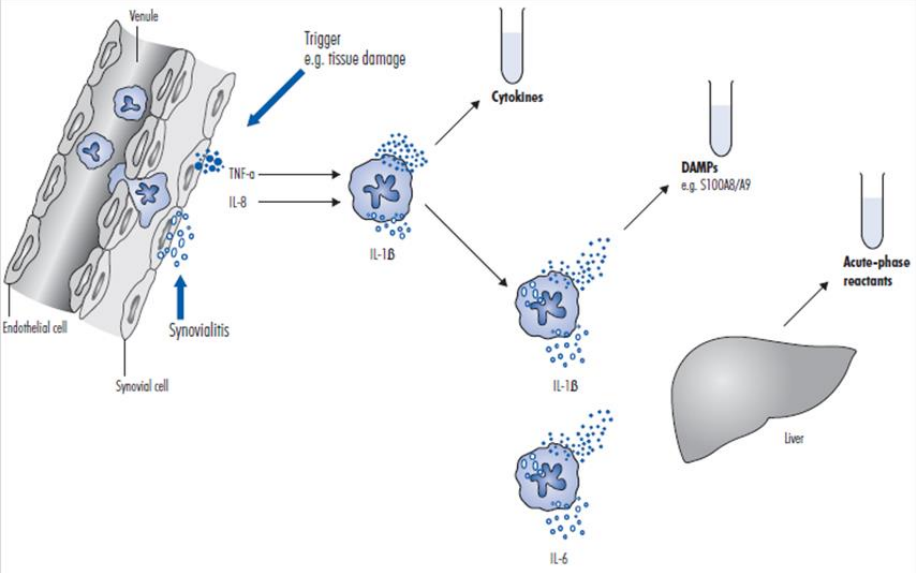


Figure 2.7. Biomarkers of inflammation linked to JIA disease pathophysiology. The innate immune system can be activated by triggers such as tissue damage in JIA. Inflammatory cells can infiltrate the joint synovium and release cytokines (e.g., TNF-α, IL-8, IL-1β and IL-6). Immune amplifiers are also released at inflamed **tissues** in response to cytokines and are more stable for laboratory measurements. Acute phase reactants, such as CRP, produced mainly in response to the circulating IL-6, is conventionally used today to measure indirect synovial inflammation (Foell and Däbritz 2011)

DAMPS, danger-associated molecular patterns; TNF, tumour necrosis factor; IL, interleukin

Biomarkers can be grouped according to their role in diagnosis, treatment, or prognosis. Three distinct groups have been identified: Type 1a, type 1b and type 2 biomarkers as listed in Table 2.2. There is no singular disease marker which defines the JIA phenotype. This depends rather on a combination of clinical biomarkers and physical examination. Type 1b and Type 2 biomarkers, relative to JIA, are discussed in section 2.4.1 below (Foell and Däbritz 2011).

Table 2.2: Description of biomarker types and functions (Adapted from Foell and Däbritz 2011)

	Type 1a	Type 1b	Type 2
Biomarker type	Disease marker	Disease subtype marker	Marker of inflammation
Function	Assist diagnosis	Assess complications and prognosis	Monitor response to treatment or predicting flares
Examples	None for JIA	ANA, RF, anti-CCP	Acute phase proteins, cytokines, S100A8/A9

ANA, antinuclear antibody; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide

2.6.1. Autoantibodies

Various autoimmune diseases result in the production of autoantibodies. The major autoantibodies related to JIA include RF, ANAs and anti-CCPs. Their involvement in diagnosis, prognosis and pathogenesis have been investigated in numerous studies. These autoantibodies form part of the type 1b group of biomarkers and can be detected using serological methods (Mahmud and Binstadt 2019; Foell and Däbritz 2011).

RF has been seen various autoimmune diseases such as adult RA, Sjögren's disease and acute rheumatic fever. Additionally, RF has also been seen in cases of infection and even in healthy individuals. In the case of JIA, RF positivity defines the subtype RF-positive polyJIA which makes up a small percentage (<5%) of all JIA cases. In addition to its usefulness as a subtyping tool, RF also has prognostic value. A higher risk for bone destruction and a more serious disease progression is evident in RF-positive polyJIA patients. Furthermore, a combination of genetic similarities and the presence of RF in this subtype have suggested its phenotypic relation to adult RA (Mahmud and Binstadt 2019).

Antinuclear antibodies are a types of immunoglobulin G (IgG) antibodies which recognise nuclear antigens. Although ANAs have no diagnostic value in JIA, they do indicate an increased risk for JIA-associated uveitis and are therefore useful for disease prognosis. Given this, it is recommended that patients testing positive for ANAs attend regular eye screening exams. The subtypes which have shown the most and least ANA positivity is oligoJIA and uJIA, respectively. In the case of PsA, ANA positivity can also be indicative of early-onset disease (Mahmud and Binstadt 2019).

Detection of anti-CCPs in patient sera has no diagnostic value in JIA either, however these autoantibodies can prove useful in predicting disease-associated risks. Resembling the relationship between RF and adult RA, anti-CCPs have also been associated with increased risk for developing adult RA in healthy individuals. In contrast, the use of RF and anti-CCP for predicting risk in JIA patients is poor, with the exception of RF-positive poly-JIA cases. In said cases, anti-CCP positivity has shown to be associated with a greater risk of a more severe disease progression. Additionally,

the presence of RF and anti-CCP in JIA cases may lead to a more aggressive treatment strategy due to increased risk of severe disease progression (Brunner and Sitzmann 2006; Mahmud and Binstadt 2019).

2.6.2. Routine Markers of Inflammation

Routinely used markers of inflammation, such as ESR and CRP, have been identified as sensitive markers of disease activity in cases of JIA despite their being non-specific and indirect indicators of inflammatory activity (Kotulska et al. 2015; Swart et al. 2016).

ESR

The ESR is the speed at which erythrocytes (red blood cells) settle in an anticoagulated blood sample. This method is an indirect measure of bodily inflammation and is accelerated by the presence of inflammatory biomarkers. In contrast to CRP, an increase in ESR in response to inflammation is a lengthier process and can remain elevated for several days before returning to normal. The results of this method can often be misleading as several factors can influence ESR such as levels of immunoglobulins, fibrinogen concentration and characteristics of erythrocytes in the plasma. Nevertheless, the test is routinely done in conjunction with CRP, and they provide consistent results 67-81% of the time when performed at the same time (Assasi et al. 2015). The predictive value of ESR for the development of uveitis in JIA patients was evaluated in a retrospective study. It was found that elevated ESR appeared to be a predictor of uveitis, and this was confirmed in 3 similar studies (Haasnoot et al. 2015). The measurement of ESR is also important when determining the disease activity score for JIA which is important for monitoring the progression of disease and treatment outcomes (Swart et al. 2016).

CRP

An acute phase protein is defined by an increase or decrease in plasma concentration in response to inflammation and falls under the type 2 group of biomarkers as mentioned in Table 2.2. The production of these proteins, for example CRP and ferritin, is regulated by liver cells, namely hepatocytes, in response to proinflammatory cytokines released by activated inflammatory cells such as monocytes and macrophages (Gabay and Kushner 1999). Both CRP and ESR have shown significant correlation ($p \leq 0.001$) with the presence of synovitis in cases of JIA (Gilliam et al. 2008).

The concentration of plasma CRP present in a sample is a direct measure of acute inflammation. Levels of CRP rise within a few hours in response to acute inflammation and revert to normal a few days later if inflammation is managed (Assasi et al. 2015).

CRP is significantly increased ($p<0.01$) in patients with acute inflammation. This was shown by Prokopec et al. when comparing subtypes of JIA patients with a healthy, age- and sex-matched control group (Prokopec et al. 2012).

Gilliam et al. also found that CRP was elevated among subtypes of JIA when compared to a healthy control group. The study evaluated 42 polyarticular, 17 oligoarticular and 9 sJIA patients as well as 18 healthy controls. The cut off for detectable CRP was ≥0.8 mg/dl. All subtypes of JIA showed detectable levels of CRP while the healthy control group showed undetectable levels (Gilliam et al. 2008).

Due to the expression of acute phase proteins in response to circulating cytokines, the measurement of CRP is not directly reflective of synovitis in cases of JIA. A more direct reflection of synovitis and acute inflammation is through measurement of damage-associated molecular patterns such as calprotectin (S100A8/A9) which has further usefulness in predicting disease flares and monitoring response to treatment (Foell and Däbritz 2011).

2.6.3. Pro-inflammatory Cytokines & Chemokines

Pro-inflammatory cytokines and chemokines are central to disease pathology in all subtypes of JIA. Elevated levels of certain biomarkers have been identified in plasma samples of most subtypes of JIA. These include TNF-α, macrophage inhibitory factor (MIF), CC chemokine ligand (CCL)-2, CCL3, CCL11, CCL22, CXC chemokine ligand (CXCL)-9, CXCL10, IL-6, IL-15, CXCL8 and IL-18, amongst others. These proteins can be measured in plasma using enzyme-linked immunosorbent assays (ELISAs) or multiplex immunoassays (e.g., Luminex platform). Analysis of the biomarker profiles, obtained from immunoassays, can be used to diagnose disease, and alter treatment. The action of pro-inflammatory biomarkers causes the characteristic joint damage of JIA; however, this destructive process can be treated using monoclonal antibodies which essentially regulate the inflammatory process (de Jager et al. 2007; Zaripova et al. 2021). Biomarkers of interest and included in the current study are discussed in more detail below.

IL-6

IL-6 is one of the most studied pro-inflammatory cytokines due to its array of functions and effects on the body. Liver cells (hepatocytes) are stimulated by IL-6 to produce the well-known acute phase protein, CRP, which is a serological marker used to test for inflammation. Arthritic patients, including those with JIA, experience inflammation of one or more joints which is associated with elevated levels of circulating IL-6. Therefore, a directly proportional relationship exists between IL-6 and CRP in a case of active disease (Woo 2002; Hahn and Kim 2010; Akioka 2019).

TNF-α

TNF is a major player of the inflammatory process due to its ability to induce the production of other pro-inflammatory cytokines in synovial tissue. Additionally, it acts on osteoblasts and osteoclasts to mediate bone and cartilage destruction (Mateen et al. 2017). A significant increase of plasma TNF-α of a JIA cohort was identified by de Jager et al. when compared to a healthy control group (de Jager et al. 2007). Due to its role in JIA pathology, TNF-α is also a major target of biologic therapy. The use of anti-TNF agents blocks the action of this cytokine and has shown great success in treating patients who do not respond to first line treatments (Barut et al. 2017).

IL-1β

The potent pro-inflammatory actions of IL-1β is similar to that of TNF-α in that it stimulates production of additional pro-inflammatory cytokines. Production of IL-1b is stimulated via innate DAMP or pathogen associated molecular patterns (PAMP) recognition. The main source of this cytokine is monocytes or macrophages (Dinarello 2018). It has been implicated in cartilage destruction and has shown correlation to disease activity. Plasma expression of IL-1β in adult RA patients is elevated (Mateen et al. 2017). Similarly, elevated IL-1β has also been implicated in the pathogenesis of sJIA. Evidence for this has been provided through administration of recombinant IL-1 receptor agonist, as a form of IL-1 blockade therapy, which has improved disease outcome in many cases (Pascual et al. 2005).

IL-2Rα (CD25)

Immune activity can potentially be measured through the quantification of soluble CD25 (sCD25). This marker correlated directly with T-cell activation and has also been identified as a potential marker for subclinical MAS in patients with sJIA (Reddy et al. 2014). A significant relationship also exists between sCD25 and the acute phase protein, CRP, in patients with JIA. In addition, significantly higher levels of sCD25 have been identified in a few subtypes of JIA when compared to a healthy control group. Mangge et al. conducted a prospective study which evaluated the usefulness of serum cytokines in monitoring disease activity and found that sTNF-receptor and sCD25 were more sensitive markers of active disease than conventional CRP and ESR (Mangge et al. 1995; Woo 2002).

IL-17

IL-17 or IL-17A, is involved in the development of chronic arthritis through its involvement in inflammation and joint damage. This biomarker enhances production of other pro-inflammatory cytokines thereby promoting inflammation of the synovium. The action of IL-17 further accelerates expression of matrix metalloproteinases which are involved in the process of cartilage destruction.

Increased plasma concentrations of IL-17 have been observed in JIA patients with active disease when compared to healthy controls (Vijatov-Djuric et al. 2017).

MIF

Macrophage inhibitory factor has a confirmed role in the pathogenesis of arthritis. This is evidenced by the action of anti-MIF treatment which dampens the severity of the arthritis by avoiding accumulation of macrophages and T-cells at the synovium (Meazza et al. 2002). The action of MIF is therefore pro-inflammatory and a significant increase in plasma expression of JIA patients has been shown when compared to a healthy control group (de Jager et al. 2007).

CCL22

This protein is referred to as macrophage-derived chemokine or CCL22 and previous studies have shown it to be present in all subtypes of JIA. These studies also confirmed that expression of this chemokine in the plasma of JIA patients was significantly higher than that of a healthy control group. Furthermore, this chemokine has been implicated in synovial inflammation which may lead to joint damage (de Jager et al. 2007).

IFN-γ

T cell cytokine, IFN-γ, regulates many aspects of the immune response such as activation of macrophages (Tau et al. 1999). Detectable levels of IFN-γ have been observed even during apparent disease remission which may be indicative of subclinical inflammation. While findings identified an apparent increase in plasma concentrations of JIA patients with active disease compared to those in remission, these results were not significant due to observed variation and needs to be investigated further (de Jager et al. 2007).

CCL11 (eotaxin)

A study comparing plasma concentrations of CCL11 between healthy controls and a JIA cohort revealed a significantly higher level of expression in the latter. This chemokine is alternatively referred to as eotaxin (de Jager et al. 2007). Eotaxin is produced by several cells such as monocytes, macrophages and lymphocytes and acts in a chemotactic capacity. Additional studies have confirmed an increase in eotaxin expression in plasma of both adult RA and JIA patients when compared to healthy controls (Syversen et al. 2008).

CXCL9

A significant difference in plasma expression of CXCL9 was found when comparing plasma biomarker concentrations of a JIA cohort with a healthy control group. The JIA cohort had higher expression of this protein which is also referred to as monokine induced by IFN-γ (de Jager et al. 2007). This chemokine is therefore upregulated in response to inflammatory conditions. Additionally,

chemokines promote recruitment of leukocytes to areas of inflammation via extravasation and can be produced by macrophages and fibroblasts (Rump et al. 2017).

CCL2

Monocyte chemotactic protein (MCP)-1 is also referred to as CCL2. This inflammatory chemokine is highly expressed in arthritic diseases such as JIA and adult RA. The production of CCL2 is regulated by other pro-inflammatory cytokines such as IL-1 and TNF-α (Rump et al. 2017). Previously, de Jager et al. showed that the plasma concentration of CCL22 is expressed at a higher level in patients with JIA when compared to a healthy control group (de Jager et al. 2007).

IL-12

IL-12 has been found to regulate the activity of lymphocytes such as T-cells and natural killer cells. In addition, IL-12 is involved in regulating the immune pathways associated with autoimmune diseases and is expressed by macrophages in the synovium. Therapies blocking IL-12 activity in murine models has dampened existing inflammation. This cytokine induces the production of additional pro-inflammatory cytokines such as IFN-γ, amongst others. Studies have confirmed elevated expression of IL-12 in the sera of JIA patients when compared to those with inactive disease or healthy controls. The use of this cytokine may therefore also be suitable for assessing disease activity (Kim et al. 2000; Woo 2002).

CXCL10 (IP-10)

Interferon gamma-induced protein (IP)-10 is also referred to as CXCL10. This chemokine, like CXCL9, has dual functions and is upregulated in response to inflammatory conditions. Therapeutics targeting chemokines and their receptors have been linked to an apparent reduction in disease severity in animal models (Rump et al. 2017). Furthermore, a trend toward significance has been identified when comparing plasma concentrations of IP-10 in JIA patients when compared to a healthy control group and must be investigated further (de Jager et al. 2007).

A statistical evaluation of plasma biomarker profiles identified a combination of biomarkers which can potentially be used to discriminate between JIA subtypes. The combination included IL-18, MIF, CCL2, CCL3, CCL11, CXCL9 and CXCL10. Additional findings also identified that even in remission, inflammatory cytokines such as IFN-γ and IL-6 were elevated when compared to expression in healthy individuals. Biomarker profiles therefore play an important role in disease prognosis, determining the need for biologic agents and for altering or removing treatment, especially during remission when subclinical inflammation may be present (de Jager et al. 2007).

2.6.4. Calprotectin (S100A8/A9)

Calprotectin is a member of the calgranulin family of proteins. It is a calcium- and zinc-binding protein with a heterodimeric structure, consisting of one light and two heavy calcium-binding chains (Vaos et al. 2013). It has many names such as S100A8/A9, myeloid-related protein (MRP)-8/-14, calgranulin A and B, L1 and cystic fibrosis antigen (García-Arias et al. 2013). Calprotectin consists of both S100A8 and S100A9 proteins which are made up of 93 and 113 amino acids, respectively. Calprotectin can play a significant role in many inflammatory processes. The monomeric structures can be released by neutrophils and monocytes, and they then form the stable heterodimer structure, S100A8/A9, which can be measured for clinical purposes. Calprotectin is a candidate marker for monitoring therapeutic responses and for diagnostic purposes in cases of inflammatory disease (Wang et al. 2018). Calprotectin is normally found in the cytosol and is secreted extracellularly by activated neutrophils and monocytes. Additionally, in the case of cell death or membrane destruction, calprotectin can also be released. This protein can be detected in different specimens such as urine, faeces, saliva, and plasma. The reference range of plasma calprotectin is <2 mg/L in healthy individuals (Stříž and Trebichavský 2003).

The emergence of calprotectin as a biomarker of inflammation has been investigated in various autoimmune or inflammatory diseases such as inflammatory bowel disease (IBD), Kawasaki disease, celiac disease, and intestinal cystic fibrosis (Vaos et al. 2013). Calprotectin is a candidate marker for monitoring inflammation in cases of juvenile arthritis and its potential as a superior marker to the traditional measurements of CRP and ESR needs to be explored further (Wang et al. 2018). It has also been demonstrated that calprotectin has increased sensitivity in relation to subclinical disease when compared to CRP and ESR (Mariani et al. 2015). The reason it may prove superior is due to the nature of the inflammation detected; acute phase proteins, such as CRP, indicate systematic inflammation as they are released by hepatocytes in response to proinflammatory cytokines while plasma calprotectin will reflect localised inflammation as it is released by activated monocytes and macrophages in the synovium and synovial fluid of affected joints. Studies have confirmed this relationship by comparing the concentrations of plasma calprotectin with disease activity and number of active leukocytes at the inflamed joint (Stříž and Trebichavský 2003; García-Arias et al. 2013). Aljaberi et al. demonstrated the correlation between disease activity and calprotectin however, this was limited to a cohort of sJIA patients only (Aljaberi et al. 2020) while Romano et al. did not find significant correlations between calprotectin and disease activity in a cohort of non-systemic JIA patients (Romano et al. 2021). Similarly, no significant correlation between these variables were identified in a recent study (Barendregt et al. 2020). It seems conflicting evidence exists describing the relationship between calprotectin and disease activity which is to be investigated in this study. Furthermore, the stability of this protein, and the ability to easily measure it in serum samples without the need for cold storage, makes this marker ideal for

diagnostic laboratories to consider as an offering to rheumatology clinics and other JIA medical centres (Moncrieffe et al. 2013; La et al. 2021).

Calprotectin is considered a DAMP, also referred to as alarmins, and act as indicators of cell damage or are secreted by activated cells. Pattern recognition receptors (PRR) present on monocytes, such as TLR4, recognise DAMPs which are secreted by activated monocytes or macrophages in response to inflammatory cytokines. Figure 2.8 illustrates the relationship between surface expression of TLR4 and calprotectin on monocytes. Due to the inflammatory nature of active arthritis in cases of JIA, the expression of calprotectin by activated monocytes and macrophages increases, thereby confirming the correlation of disease activity with plasma calprotectin levels (Erchen et al. 2009; Frosch et al. 2000). Romano et al. also demonstrated that calprotectin correlated significantly with infiltrating monocytes at the site of joint damage in patients with JIA (Romano et al. 2021).

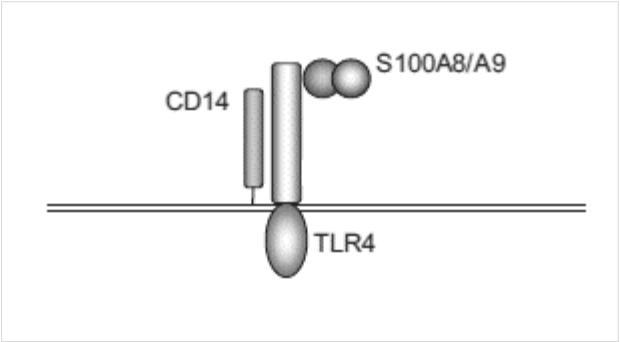


Figure 2.8. Calprotectin (S100A8/A9) is a DAMP recognised by TLR4 expressed on monocytes (adapted from Ehrchen et al. 2009)

Plasma concentrations of soluble calprotectin have been shown to be significantly decreased in JIA patients who were in remission as compared to those with active disease. The results of monitoring calprotectin may also be predictors of relapse and thus detect sub-clinical inflammation. Furthermore, the benefits of recording the levels of calprotectin in inactive JIA cases could serve as a reference range for remission and therefore act as evidence to stop treatment. Positive correlations between plasma calprotectin and ESR have also been observed (Vaos et al. 2013; Bojko 2017). Boyko et al. recently demonstrated direct correlations between calprotectin, JADAS scores, ESR and CRP in JIA patients. The relationship between sJIA and elevated calprotectin was also confirmed in this study and thereby highlighted the usefulness of blood calprotectin in the process of diagnosing sJIA (Boyko et al. 2020).

Study Rationale

There is a major gap in JIA research when it comes to studies done in South Africa, as evidenced by the lack of publications on the topic. The aim of the study was to contribute towards filling this gap by investigating the role of calprotectin, in relation to disease activity and inflammation, in a cohort of JIA patients in the Western Cape, South Africa. In addition, the study also attempted to confirm the role of calprotectin as a marker of inflammation and subclinical disease in a diverse population cohort of JIA patients and to determine whether it may be a superior marker to conventional measurements of CRP and ESR in disease monitoring. Calprotectin (S100A8/A9) has been studied in many inflammatory disorders and has previously been correlated to inflammation. Subclinical inflammation may also be detected with the increased sensitivity afforded in measuring calprotectin. Conflicting evidence exists describing the relationship between disease activity and calprotectin, this was investigated for future influence on disease monitoring strategies. Combining evaluation of calprotectin with other biomarkers, diagnostic tests, and monocyte distribution, provided additional data for better interpretation of the findings.

Aims and Objectives

The aim of the study was to investigate the sensitivity and specificity (compared to that of conventional markers CRP and ESR) of plasma calprotectin as a biomarker for monitoring inflammation and response to treatment in cases of JIA recruited at the Tygerberg Paediatric Rheumatology Clinic, Western Cape, South Africa. The action of calprotectin in disease activity is linked to the binding of monocytes, via TLR4, thereby activating the autoimmune response. This study also aims to show this relationship between monocytes and calprotectin through measurement of monocyte numbers and subsets, TLR4 binding protein and calprotectin concentration. The study further aims to identify the role of calprotectin in predicting the risk in disease relapse by measuring levels of calprotectin and monitoring disease status throughout the sampling period and shortly after the sampling period ended.

This pilot study was divided into three parts; Part 1 was a cross-sectional evaluation of plasma calprotectin and other JIA-related inflammatory biomarkers in JIA patients attending TBH and included patients at various stages of disease i.e., newly diagnosed, undergoing treatment, or in remission on treatment. Part 2 was a longitudinal evaluation of plasma calprotectin and inflammatory biomarkers in patients, recruited during part 1 (JIA cohort), who attended routine follow ups approximately six months later. Originally, it was planned that treatment naïve patients would be included as well and followed throughout treatment to observe changes in inflammatory profiles over time; however, these were not plentiful at the time of recruitment. Part 3 of the study was then included and shifted toward monitoring treatment efficacy over time and investigating any other differentiators of inflammatory or disease activity within stratified analysis groups.

I. The objectives for Part 1 of the study included:
    a. Recruiting JIA patients at visit A (diagnosed at ≤ 16 years old and undergoing treatment or at diagnosis) and quantifying their levels of plasma calprotectin.
    b. Collecting clinical data from patient files and the NHLS Trakcare system relating to diagnosis, disease activity, number of active joints, medication lists, ESR, CRP, differentials, and full blood counts.
    c. Comparing plasma calprotectin levels to standard diagnostic laboratory markers of inflammation (ESR & CRP) and several other key inflammatory or JIA-associated markers measured in plasma.
    d. Showing the relationship of monocytes (numbers, subsets distribution, activation status and TLR4 [calprotectin receptor] expression) to plasma calprotectin levels in order to observe monocyte activation by TLR4 binding of calprotectin.

II. In a sub cohort selected from Part 1, the objectives for Part 2 of the study included:
    a. Quantifying levels of plasma calprotectin and JIA associated markers in patients attending follow ups (visit B) roughly six months later (patients must have been recruited in Part 1).
    b. Demonstrating changes in inflammatory markers, monocytes, and disease activity in response to treatment, over time from visit A to B.
    c. Investigating the role of calprotectin in predicting risk of relapse after visit A and visit B.

III. Part 3 of the study included:

    a. Identifying which inflammatory markers showed any evidence of changing over time given the confounding factor of ongoing treatment.
    b. Stratifying participants based on markers discovered in objective a, to identify possible differentiators of inflammatory activity.
    c. Stratifying patients based on their disease status to identify possible differentiators of disease activity.

Chapter 3 Methodology

3.1 Participant Recruitment and Sample Collection

The study was approved by the Health Research Ethics Committee (HREC), Faculty of Medicine and Health Sciences at Stellenbosch University. Approval to conduct this research at TBH was subsequently obtained from the TBH Manager, Dr GG Marinus. The ethics reference number is S19/05/101 and was originally approved in 2019 with subsequent renewal in 2020 and 2021.

A cohort of 22 JIA patients were recruited and/or monitored at the TBH Paediatric Rheumatology Clinic over a period of almost one year starting in March 2020 to May 2021. The originally planned collection timeline was significantly negatively impacted by the coronavirus disease (COVID)-19 pandemic. Patients were identified and selected as potential participants in this research study by the attending paediatric rheumatologist, Dr Deepthi Raju Abraham, a study co-investigator. Patients were selected based on a JIA diagnosis obtained through clinical examination and standard laboratory testing prior to attending the TBH Paediatric Rheumatology Clinic where blood was to be drawn for further specialised testing. No other selection criteria were defined, and sampling was done opportunistically over a period of several months, i.e., when a JIA patient attended the clinic. Blood collection was done by nursing staff in C3A, on the 3rd floor of TBH.

Eligibility for participation in the study included a diagnosis of JIA, in the absence of any other inflammatory disorder, and an age of 16 years or younger at the time of diagnosis. Recruited patients were from the surrounding areas of TBH, which is one of two centres that service all JIA patients in the Western Cape. Patients enrolled in this research were either initiating (n=1) or currently undergoing treatment (n=21) for JIA. Treatment regimens are detailed in section 4.1.1. Essential demographic and clinical details for each patient were obtained from patient files. These included: age, diagnosis and ILAR defined disease subtype, number of active joints, list of medications, time on treatment, disease activity and whether the child experienced a disease flare following each visit. Disease activity was indicated in patient files and was based on Dr Abraham's assessment of the child, their JADAS score, and overview of their routine laboratory investigations. Disease activity was classified as either low, moderate, or inactive. Disease flare was evident at visit B, following visit A, for those returning participants who showed signs of inflamed joints (evidenced by elevated ESR or CRP) together with clinical identifiers (joint swelling, heat, and/or pain) as noted by Dr Abraham. Similarly, flares after visit B were indicated by Dr Abraham and were characterised by increased inflammation of the joints with or without ongoing treatment. The cohort recruited for this study would be assessed at 3 and 6-months following their initial blood draw for longitudinal analysis.

Patients, parents, or guardians were required to read and sign assent (Appendix A) and consent (Appendix B) forms following a discussion about the research with the researcher. A flyer (Appendix

C) was also provided with some more information about JIA, these could be taken home and shared with friends and family to bring awareness to this rare disease.

3.1.1. Routine Laboratory Testing and Sample Collection

All eligible consented patients attending the regular Tuesday morning paediatric rheumatology clinic (ward C3A, TBH) had no more than 14 mL of blood drawn via venepuncture. Total of 9 mL was used for routine laboratory testing which was done in order to quantify and assess levels of CRP, ESR, FBC and differential blood cell count, all of which were performed at the National Health Laboratory Service (NHLS). Further testing for disease subtyping e.g., RF and ANAs was also performed as required. Routine test results for each patient were subsequently obtained from the NHLS TrakCare database through Prof Richard Glashoff, primary investigator and NHLS scientist.

The remaining 5 mL of whole blood drawn at the clinic, was transferred into labelled ethylenediaminetetraacetic acid (EDTA) Vacuette tubes (Greiner Bio-One, Kremsmünster, Austria) provided to nursing staff. This process was repeated at a follow up visit within 3 – 6 months following the initial sample collection.

3.1.2. Plasma Storage

Whole blood samples collected at the clinic were aliquoted for use in separate experiments and were processed within 4 hours of collection. A total of 0.5 mL of each sample was set aside for phenotypic analysis of cell populations by flow cytometry. The remaining volume of each sample was centrifuged at 2200 relative centrifugal force (RCF) for 15 minutes using the Heraeus Megafuge 2.0 R (Heraeus Group, Hanau, Germany). Following centrifugation, the top plasma layer was collected and stored in 350 µL aliquots in the -80°C freezer for later cytokine and biomarker analysis.

3.2 Monocyte Phenotypic Assessment by Surface Marker Flow Cytometry

Preparation of whole blood for analysis by surface marker flow cytometry was performed within 4 hours of sample collection. Monocyte numbers, subset distribution, activation status and expression of TLR4, the calprotectin receptor, were assessed.

3.2.1. Panel Design (6-colour)

A 6-colour panel was designed for the surface marker flow cytometry using the BD Horizon™ Guided Panel Solution (GPS) tool and guidance from BD application specialists. Six monocyte markers were of interest for this study and were conjugated to the following fluorochromes: CD14-APC, CD16-Brilliant Violet 421 (BV421), CD36-Phycoerythrin (PE), CD64-Brilliant Violet 510 (BV510), CD86-

Brilliant Blue 515 (BB515) and CD284-Brilliant Blue 700 (BB700) also referred to as toll-like receptor 4 – Brilliant Blue 700 (TLR4-BB700). These fluorochrome-conjugated antibodies, summarised in Table 3.1, were manufactured by BD Biosciences (BD Biosciences, CA, USA).

Table 3.1: Description of fluorochrome-conjugated monoclonal antibodies (mAb) used for the 6-colour surface marker flow cytometry method.

mAb (monocyte)	Fluorochrome	Clone	Reagent Description	BD Catalogue Number
CD16 (FcγRIII)	BV421	3G8	BD Horizon™ BV421 Mouse Anti-Human CD16	562874
CD86	BB515	2331	BB515 Mouse Anti-Human CD86	564544
CD284 (TLR4)	BB700	TF901	BD OptiBuild™ BB700 Mouse Anti-Human TLR4 (CD284)	745946
CD14	APC	MφP9 (also known as MφP-9)	APC Mouse Anti-Human CD14	340436
CD64	BV510	10.1	BD Horizon™ BV510 Mouse Anti-Human CD64	563459
CD36	PE	CB38 (also known as NL07)	BD Pharmingen™ PE Mouse Anti-Human CD36	555455

3.2.2. Sample Preparation

Whole blood samples collected from patients attending the TBH paediatric rheumatology clinic were immediately transported to the laboratory for processing and storage procedures. Sample preparation for flow cytometry required only 0.5 mL of the total 5 mL collected from each patient – the remaining volume was processed for plasma storage as discussed in section 3.1.2.

A volume of 100 uL whole blood was transferred to a flow tube and then stained with the appropriate volumes, determined via titration (i.e., section 3.2.3) of each antibody (Table 4) together with 50 uL brilliant stain buffer (BD Biosciences, CA, USA). The sample was vortexed and incubated in the dark, at room temperature, for 30 minutes. Red blood cells were lysed using 1.5 mL of a 1X BD FACS™ lysing solution (BD Biosciences, CA, USA). The sample was again incubated in the dark, at room temperature, for approximately 20 minutes. Following this, the sample was centrifuged at 2000 RCF for 5 minutes and the supernatant was discarded. The flow tube was then inverted, and the remaining fluid was gently blotted onto paper towel. The remaining pellet was resuspended and washed using 1 mL of staining medium (2% foetal bovine serum in phosphate-buffered saline) and vortexed. The cell suspension was centrifuged again at 2000 RCF for 5 minutes and the supernatant was discarded. The cells were washed once more before they were resuspended in 400 uL of staining

medium, vortexed and then stored at 4°C. The sample was analysed immediately, or within 18 hours, on the flow cytometer.

3.2.3. Determining Optimal Monoclonal Antibody Staining Volumes

The optimal concentration of each antibody was determined by standard antibody titration procedures in order to ascertain the lowest volume possible to achieve best staining and signal detection, determined by separation of negative and positive populations when analysed by flow cytometry.

For the titration procedure, a 2-fold dilution series was performed for each antibody. The manufacturers' recommended staining dilution (calculated from recommended volume) was 1:20, so cells were stained with 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640 dilutions. Each antibody was tested at these various dilutions and then analysed by plotting the stain index vs antibody volume.

The stain index was determined for each antibody and is defined as the ratio of the separation between the positive and negative populations, divided by 2 times the standard deviation of the negative population. It can be calculated using the following formula:

$$\text{Stain Index } (\Delta) = \frac{MFI\ pos - MFI\ neg}{2\ x\ SD\ neg}$$

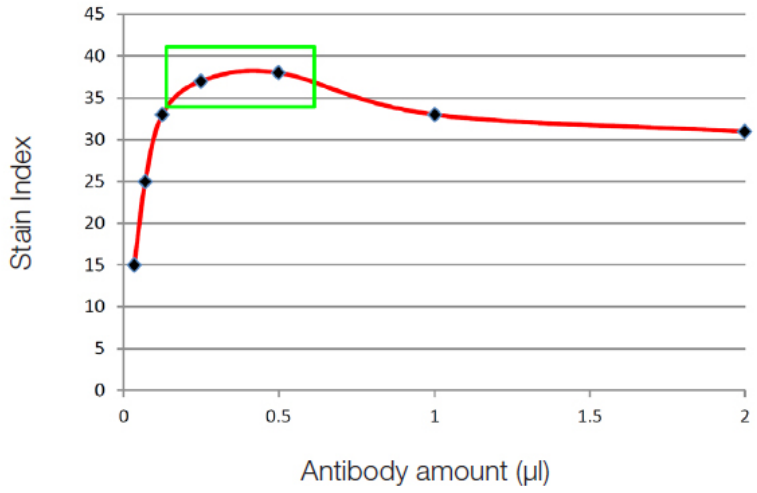


Figure 3.1. Example titration curve for standard index vs antibody volume. The titration curve allows for selection of the lowest possible volume used to avoid low affinity binding (Bio-Rad, 2022).

The stain index was plotted against final antibody volume (Figure 3.1) to observe the optimal staining volume for each antibody (Table 3.2). The optimal antibody volume can be chosen at the best stain index.

Table 3.2: **Optimal volumes (µL) for cell staining procedure.**

mAb (monocyte)	Fluorochrome	Manufacturers Recommended Volume (µL)	Optimal Volume (µL)
CD16 (FcγRIII)	BV421	5	0.156
CD86	BB515	5	0.625
CD284 (TLR4)	BB700	5	0.625
CD14	APC	20	10
CD64	BV510	5	2.5
CD36	PE	20	10

3.2.4. Instrument Set-up

All samples were analysed using the BD FACSCanto™ II (BD Biosciences, CA, USA) located in the BD-CAF Flow Cytometry Centre, Educational Building, Room 5033, Faculty of Medicine and Health Sciences, Stellenbosch University. The FACSCanto™ optics system features three lasers: a blue (488 nm), red (633 nm) and violet (405 nm) laser. Instrument set-up, data acquisition and analysis were done using the BD FACSDiva software.

At each use of the instrument, the cytometer performance was assessed using BD cytometer setup and tracking (CS&T) beads (BD Biosciences, CA, USA). The CS&T reagent contains equal concentrations of polystyrene beads of differing intensity (dim, midrange and bright) and varying excitation and emission wavelengths. The results generated by processing these beads in the cytometer determine optimal application settings for reproducible performance.

3.2.5. Compensation

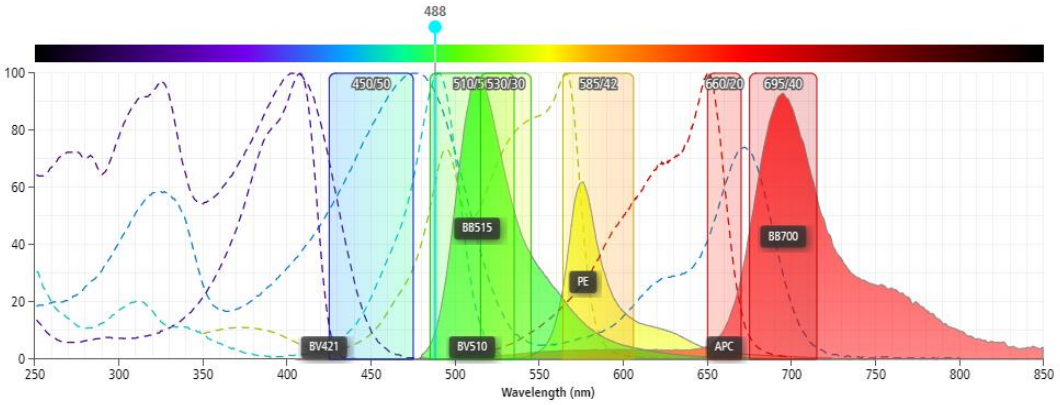
Compensation for spectral overlap is necessary to account for spill over of fluorochromes into the various detectors and a lack of compensating can lead to misinterpretation of the data.

Figure 3.1 represents the fluorochrome emission spectra for the panel chosen for this study. These graphs were generated using the BD Spectrum Viewer tool online (BD Biosciences 2021 BD Spectrum Viewer (bdbiosciences.com)).

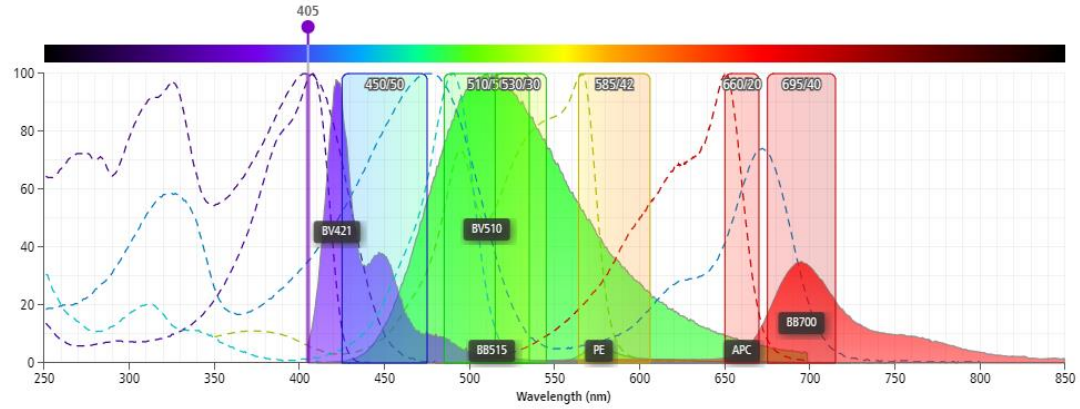
Compensation was performed by using BD™ CompBeads (BD Biosciences, CA, USA). Each fluorochrome was mixed with one drop of anti-mouse Ig, kappa (κ) and one drop negative control compensation beads together with 100 uL of staining buffer.

The optimal staining volumes determined for each fluorochrome was added to the compensation mixture and incubated for 30 minutes, in the dark, at room temperature. Following incubation, the beads were washed and resuspended in 400 uL staining buffer in preparation for acquisition on the BD FACSCanto™ II (BD Biosciences, CA, USA).

Blue 488nm laser



Violet 405nm laser



Red 633nm laser

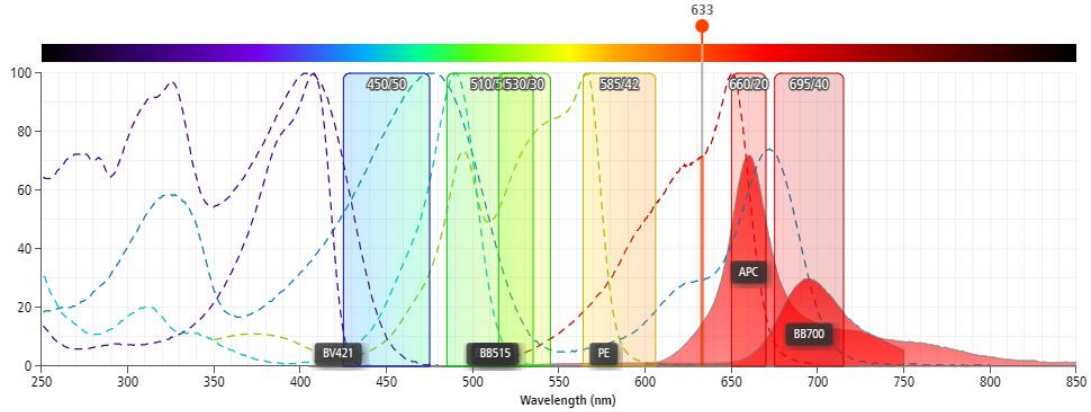


Figure 3.2. Graphs to show the spectral overlap among the fluorochromes chosen for this experiment. The absorption and emission spectra for each fluorochrome are shown for blue, violet, and red lasers above. The colourful rectangles within the emission spectra (solid lines) represent the portion detected by the band pass filters. There is clear spill over of BB515 and BV510 only for the chosen fluorochromes.

3.2.6. Fluorescence Minus One (FMO) Controls

FMO controls are samples stained with all fluorochrome-conjugated antibodies but one. These are used to accurately observe positive and negative expression of each marker (i.e., to define the cut-off between negative and positive populations). A whole blood sample from a patient was used to create the FMO controls for each of the markers. Population gates were used in order to distinguish between certain cell types for later analysis. Monocyte subset gates are well described, and this allowed for easy distinction of positive and negative subset populations, Monocyte populations can be divided into classical (CD14++CD16-), non-classical (CD14+CD16++) and intermediate (CD14++CD16++) based on their CD14 and CD16 receptor expression profiles. An example of the FMO control for CD14 is shown in Figure 3.3.

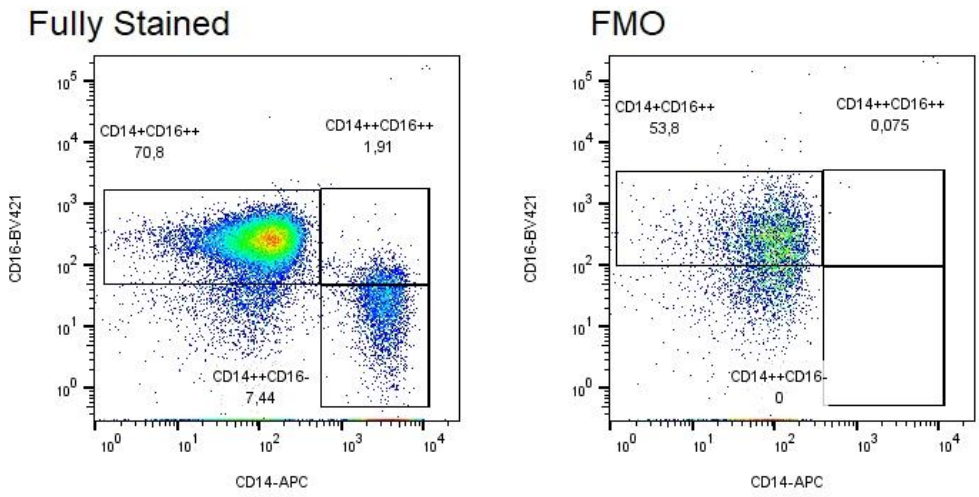


Figure 3.3. FMO control gating for CD14. Samples stained fully have positive populations of CD14 cells i.e., the classical (CD14++CD16-) and non-classical (CD14+CD16++) monocyte subsets. The FMO stained sample shows no CD14 positive monocyte subsets. FMO controls allow for more accurate distinction of positive and negative populations.

3.2.7. Gating of Monocyte Subsets and Marker Expression

Prepared samples were acquired on the BD FACSCanto™ II and data was observed using the BD FACSDiva software.

Gating of the monocyte populations was defined prior to sample acquisition and is illustrated in Figure 3.3. Firstly, a ‘time gate’ was set up using SSC vs. Time to ensure a steady rate of acquisition over time. Secondly, a ‘not monocyte gate’ was set up using SSC vs. CD16-BV421 to remove all highly positive staining neutrophils from the population of interest. Thirdly, the entire monocyte population was gated on using SSC vs. FSC. Single cells were then gated using FSC-Height vs. FSC-Area to remove any doublets. Following this, all three monocyte subsets (classical, intermediate, and non-classical) could be distinguished by plotting CD16-BV421 vs. CD14-APC.

Within each monocyte subset population, expression of CD36 and CD86 could be determined by plotting CD36-PE vs. CD86-BB515. Additionally, expression of CD64 and TLR4 could be determined on each subset population by plotting CD64-BV510 vs. TLR4-BB700 (see gating strategy illustrated on Figure 3.4 below).

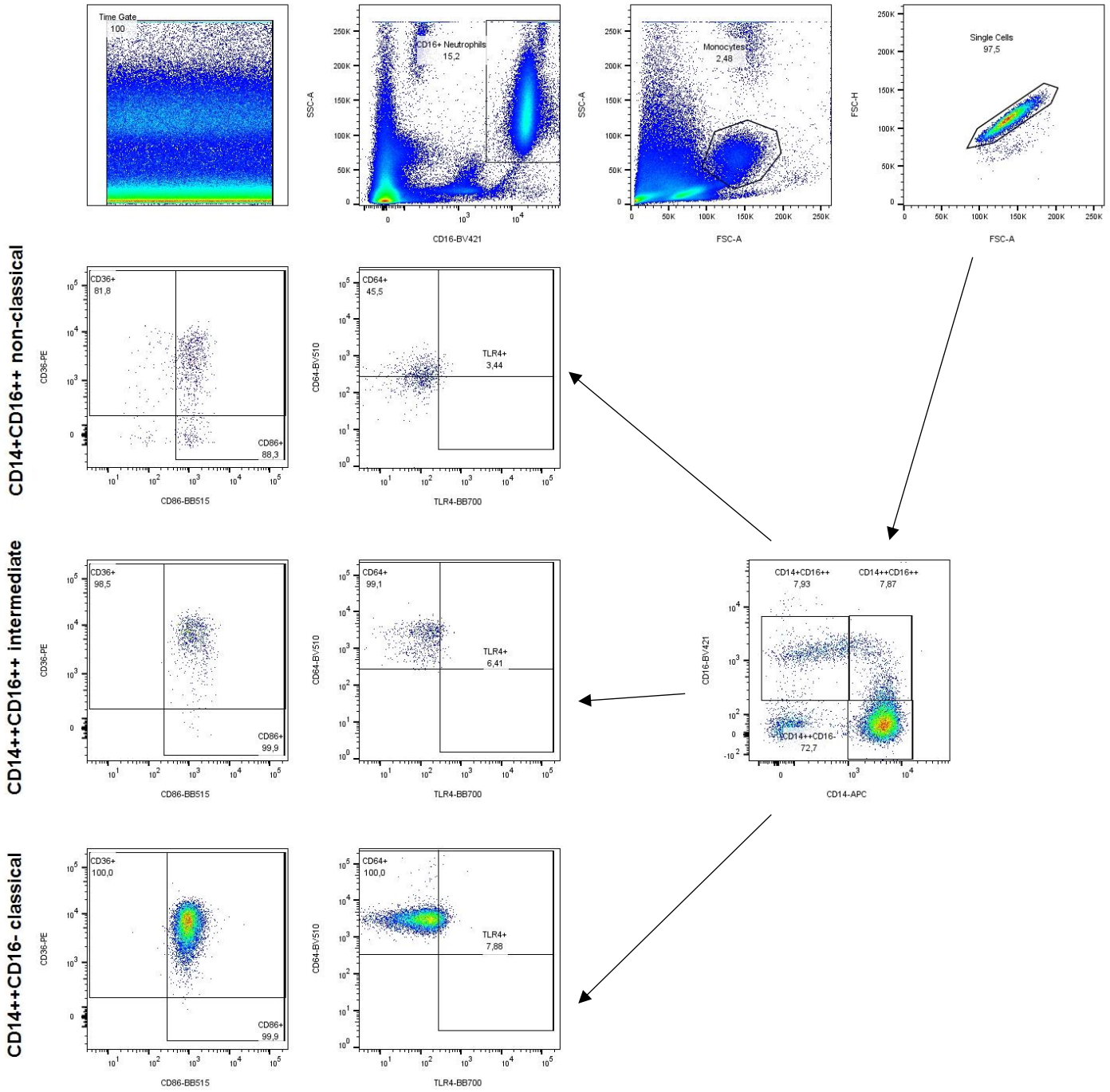


Figure 3.4. Gating strategy used to observe monocyte subsets and surface marker expression. Highly positive CD16 neutrophils are excluded using a 'not gate'. The monocyte population was isolated using SSC vs. FSC. Doublets are excluded and monocyte subset populations are observed using CD16-BV421 vs. CD14-APC. CD36 and CD86 expression were observed on all subsets using CD36-PE vs. CD86-BB515. Expression of CD64 and TLR4 were observed on all subsets using CD64-BV510 vs. TLR4-BB700. Gating graphics generated by FlowJo ® (Version 10).

Patient samples were acquired with a total of 10 000 events being recorded in the monocyte gate. Data was compiled on BD FACSDiva software was exported to .fcs files and analysed using FlowJo® (Version 10). Percentage expression of monocyte populations and surface markers were exported to MS Excel for statistical analysis using GraphPad Prism Software (Version 9).

3.3 Inflammatory Biomarker Detection

Assays to detect inflammatory biomarkers were performed using stored plasma isolated from patient blood samples collected during routine clinical visits at the TBH paediatric rheumatology clinic. Stored plasma was kept at -80°C for approximately one year until all sampling for this study was completed – this was done in order to batch samples for each immunoassay; Calprotectin was measured using ELISA and the remaining markers by Luminex.

3.3.1. Detection of Calprotectin (S100A8/A9)

A Human S100A8/A9 Heterodimer Quantikine® ELISA kit (R&D Systems, MN, USA) was used to detect and quantify levels of the calprotectin protein (S100A8/A9) in each of the stored plasma samples.

The assay was performed according to the manufacturer's instructions. In short, all reagents were bought to room temperature and plasma samples collected from the -80°C freezer were thawed. Preparation of wash buffer, substrate solution and standards were required. The standard concentrations for S100A8/A9 were 40 ng/mL, 20 ng/mL, 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL and 0.625 ng/mL. Assay diluent was added to each well of the microplate. In addition, standards or samples were added in duplicate following the plate layout. The microplate was covered and incubated for 2 hours at room temperature on a horizontal shaker. Following incubation, wells were washed and aspirated using the Bio-Rad ImmunoWash™ 1575 microplate washer in preparation for the next step. The S100A8/A9 conjugate was added to each well and the microplate was incubated for 2 hours at room temperature on a horizontal shaker. Another wash step was performed before adding the substrate solution to each well and incubating for 30 minutes in the dark. A stop solution was added to each well and a colour change was observed. The optical density (OD) of each well was measured within 30 minutes of adding the stop solution using the Bio-Rad iMark™ Microplate Reader at 450 nm (595 nm correction wavelength).

Calculation of results

Duplicate OD readings for each standard and sample were averaged, and the average zero standard OD was subtracted. A standard curve was generated using the mean absorbance (y-axis) and concentration (x-axis) of each standard as illustrated in Figure 3.3. The curve was created using Bio-Rad Microplate Management software in order to extrapolate a four-parameter logistic (4-PL) curve-fit.

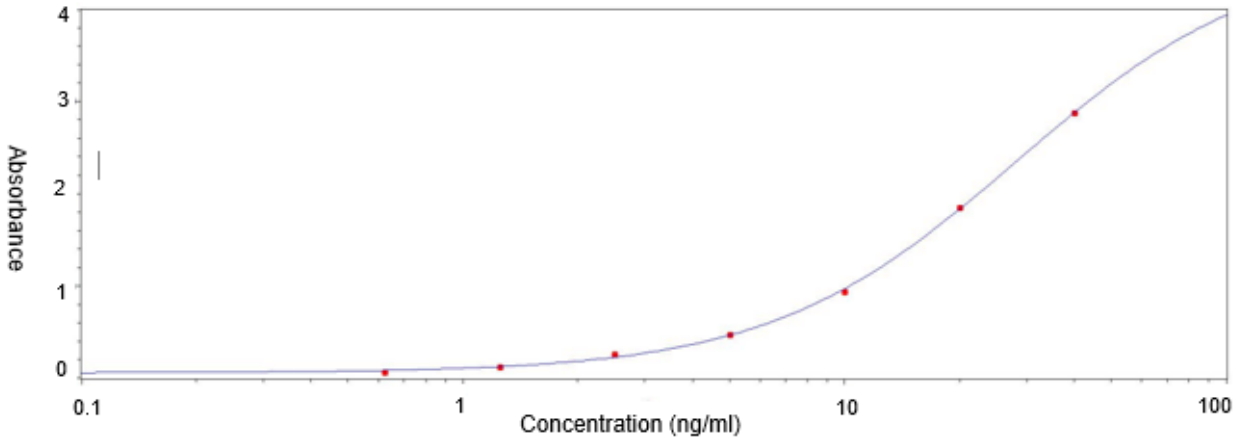


Figure 3.5. Four-parameter logistic (4-PL) curve fit for the human S100A8/A9 heterodimer ELISA standard concentration and corresponding OD readings (Chi²=0.005; r²=1.000; RMS=0.023). The curve was generated using the Bio-Rad Microplate Management software. The Chi-squared test demonstrates that there was little difference in what was expected and what was observed (Chi²=0.005). The r-squared value (r²=1.000) indicates that the regression prediction perfectly fits the data. The root mean square (RMS) represents the area under the curve (RMS=0.023).

The standard curve was used to calculate the relative concentration of each sample. These results were then multiplied by the dilution factor to obtain the final sample concentration used for analysis.

3.3.2. Detection of Additional Biomarkers

Twelve additional biomarkers were selected based on their role in JIA pathogenesis. These specific 12 were chosen following review of the literature and additionally to investigate their role, if any, in the disease monitoring process by comparing their expression to that of standard inflammatory markers such as CRP and ESR, which are currently used in the clinic.

Two Human Premixed Multi-Analyte Luminex® Assay kits (R&D Systems, MN, USA) were used to detect and quantify levels of twelve different inflammatory biomarkers of interest. These will be referred to as kit A and kit B. Kit A was used to quantify levels of sCD14. Kit B was used to quantify levels of CCL2, CCL11, CD163, CXCL9, CCL3, CCL22, CD25, CXCL10, IL-1β, MIF, IL12, TNF-α and IFN-γ.

The assays were performed according to the manufacturer's instructions. Briefly, all reagents were bought to room temperature and plasma samples collected from the -80°C freezer were thawed. Preparation of wash buffer, microparticle cocktail, biotin-antibody cocktail, streptavidin-PE and standards was required. Standards and samples were added, in duplicates, to each microplate. The microparticle cocktail was then added to each well and microplates were placed on the horizontal plate shaker and incubated in the dark, for 2 hours, at room temperature. The wells were washed and aspirated using a magnetic plate washer. The diluted biotin-antibody cocktail was added to each well and the microplates were placed on the horizontal plate shaker and incubated in the dark, for 1 hour, at room temperature. The wash step was repeated, and the diluted streptavidin-PE was added to each well before incubating for a further 30 minutes on the shaker. Another wash step was performed before resuspending the microparticles in wash buffer and incubating for only 2 minutes on the shaker. Plates were read using the Bio-Rad Bio-Plex™ 200 System.

Calculation of results

Duplicate readings were averaged for each standard and sample and the mean blank median fluorescence intensity (MFI) was subtracted. Standard curves were plotted for each biomarker using a 5-PL curve-fit as illustrated in Figure 3.6. Concentrations of samples were calculated using the standard curves and results were multiplied by dilution factors where appropriate.

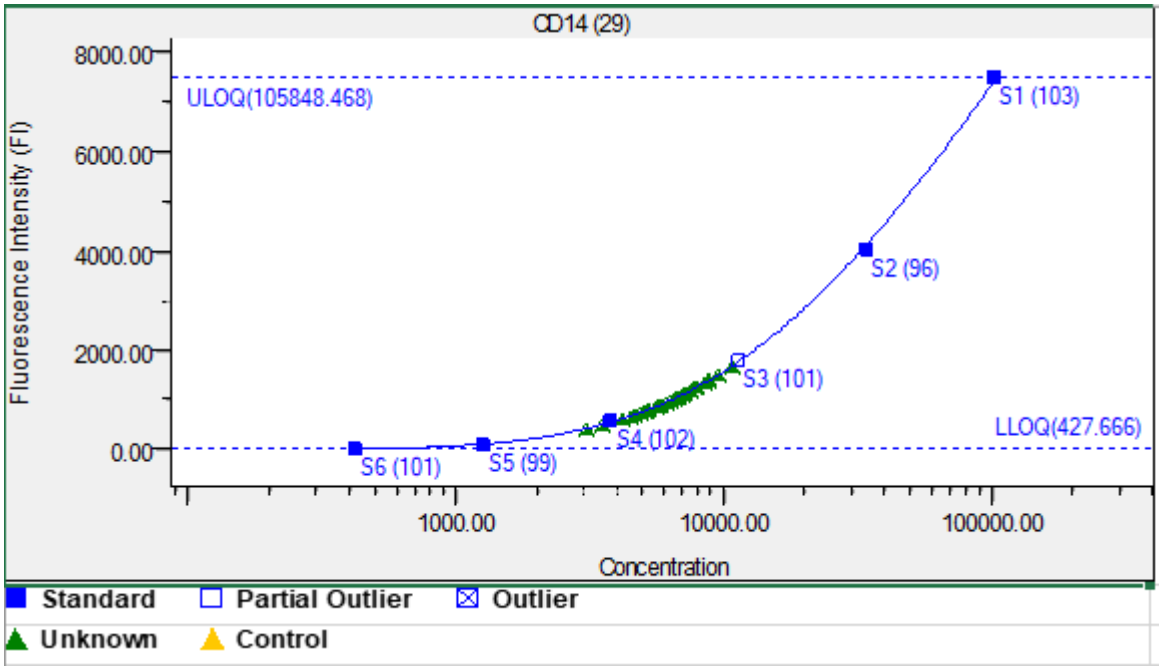


Figure 3.6. Standard curve plotted using the 5-PL curve-fit for CD14. The standards (S1-S6) were plotted at their known concentrations: 105 848pg/ml, 32 933pg/ml, 11 544pg/ml, 3 867pg/ml, 1 251pg/ml and 428pg/ml. Unknown symbols in green represent the detectable samples which all fall between 3 867-11 544pg/ml. Figure generated by analysis software.

3.4 Stratification of the Cohort

The recruited cohort was stratified to investigate any differences in inflammatory profiles between active and inactive disease phenotypes. Disease activity, determined by Dr Deepthi Raju Abraham, for each participant was recorded from patient folders at the TBH Paediatric Clinic. Participants with low or moderate disease activity were included in the active disease group, while those with inactive disease were grouped together.

The cohort was also stratified by measures of ESR since this marker showed the most evidence for fluctuating inflammation even with ongoing treatment. The low ESR group consisted of normal ESR measurements, i.e., those that were within the normal range for a healthy child (0-10 mm/hr) while the high ESR group consisted of those with an ESR measurement above the normal range for healthy children (> 10 mm/hr). The groups were compared to identify any other mediators that may have been useful in distinguishing those with some ongoing inflammatory activity vs those without.

3.5 Statistical Analysis

All study participant data was collected, and a database was created using a Microsoft Excel Spreadsheet (Microsoft, USA). The database included participant clinical information, routine diagnostic test results (CRP, ESR, FBC and differential), cellular data compiled on FlowJo®, S100A8/A9 ELISA data and Luminex biomarker data (CCL2, CCL11, CD163, CXCL9, CCL3, CCL22, CD25, CXCL10, IL-1β, MIF, IL12, TNF-α and IFN-γ). Routine tests, ELISA results, and Luminex data represented continuous variables which were listed in the analysis database. The database was used for statistical analysis in the cross sectional and longitudinal objectives of this study.

Statistical analysis was performed using GraphPad Prism Software (Version 9.0, GraphPad, CA USA). Non-parametric data was shown on a modified box-and-whisker plot illustrating only the range, median and interquartile ranges. A cross sectional analysis was performed to observe any relationships between variables at baseline (visit A) and then also at follow up (visit B). Significant relationships between variables were calculated using the Pearson correlation test. The relationships between the main markers (CRP, ESR, and S100A8/A9) were illustrated using simple linear regression. Longitudinal analysis was performed to observe a significant change over time from visit A to visit B. This was done using either the Wilcoxon sign-ranked test which tested for significance between two related groups, or Mann-Whitney test which tested for significance between two unrelated groups. The Kruskal-Wallis test followed by Dunn's multiple comparisons test was used to perform multiple tests at once amongst unrelated groups. Findings were considered statistically significant where p-values were less than 0.05 ($p<0.05$).

Chapter 4 Results

This chapter consists of four sections. The first (4.1) describes the participants and clinically relevant information. The second (4.2) summarises the data collection results and the third (4.3) analyses the relationships between calprotectin and routine markers of inflammation. The next three sections (4.4-4.6) investigate the relationship between calprotectin and other inflammatory biomarkers, monocytes, and disease activity. The last section (4.7) outlines the case studies to be discussed.

4.1 Participant Recruitment

A total of 22 participants with confirmed JIA were recruited to this study (visit A). Of these 22 participants, only 13 (59%) were included in the follow up (visit B), roughly six months after their initial visit. The cohort of 22 participants was made up of 9 (41%) experiencing active disease and 11 (50%) with an inactive disease status, all of whom were undergoing treatment. Only 1 participant was recruited as 'treatment naïve' while experiencing active disease and will be discussed further in Chapter 5. Disease status for 1 participant was unknown due to a missing file which detailed this information (note that other information was still available due to the NHLS Trakcare database, and our immunoassays, therefore this participant was not excluded). All participants were numbered (1-22) in conjunction with either A or B, representing initial visits (1A-22A) or routine follow ups (1B-22B), respectively.

Demographics and Clinical Information

Table 4.1 summarises the clinical information relating to the participants in this study. The clinical backgrounds are reported for baseline (visit A), and routine follow up clinical visits (visit B). Individual participant data is detailed in Appendix D.

All recruited participants were diagnosed at or before the age of 16 with persistent arthritis and were therefore within the ILAR's definition for JIA. There was a clear gender bias with 18 (82%) female and 4 males (18%) recruited to the study at visit A. Of the 22 participants recruited, 9 (41%) were diagnosed with oligo-JIA, 8 (36%) with RF+ or RF- poly-JIA, and 5 (23%) with sJIA. There were no participants diagnosed with ERA, PsA or uJIA. All participants were undergoing treatment upon recruitment (except for Participant 16) to manage ongoing inflammation or in the process of weaning medication while maintaining clinical remission. In addition, all participants were from areas adjacent TBH, which represents a similar socio-economic background.

Table 4.1: Clinical features of participants with blood drawn at visit A and visit B

	Visit A Median (IQR)	**Visit B** Median (IQR)
Total number of participants	22	13
Gender (F:M)	18:4	11:2
Age at recruitment	14.5 (11.25 - 15)	15 (10 - 15)
JIA subtypes		
oligoJIA	9	6
polyJIA (RF +/-)	8	4
sJIA	5	3
Number of active joints	0.5 (0 – 1.25)	0 (0 – 1)
Disease status		
Inactive disease activity	11	9
Low disease activity	3	2
Moderate disease activity	7	2
Medications		
MTX	16	13
Prednisone	4	2
Sulfasalazine	3	1
Tocilizumab	3	2
Hydroxychloroquine	7	8
Leflunomide	1	0

The most prescribed medication at both visits was MTX accounting for 73% and 100% of all participants' medication lists at visit A and visit B, respectively.

4.2 Data Collection

Table 4.2 summarises the calprotectin, blood counts and differentials of the cohort at visit A and visit B. Details for each individual participant is tabulated in Appendix E. Calprotectin was within the normal range of 127 - 1395 ng/mL (NHLS, TrakCare Database) at visit A (Median: 628.6ng/mL; IQR: 422.85 – 918.95 ng/mL) and visit B (Median: 1069.1 ng/mL; IQR: 511.8 – 1379.5 ng/mL). Additionally, CRP was within the normal range of < 10 mg/mL (NHLS, TrakCare Database) at visit A (Median: 1 mg/mL; IQR: 0.9 – 1.75 mg/mL) and visit B (Median: 1 mg/mL; IQR: 1 – 5 mg/mL).

The total number of monocytes at visit A (Median: 0.39 x 10^9/L; IQR: 0.27 – 0.45 x 10^9/L) and vsit B (Median: 0.34 x 10^9/L; IQR: 0.21 – 0.46 x 10^9/L) for all samples fell within the normal range which is between 0.2 - 0.8 x 10^9/L (NHLS TrakCare Database).

All other markers listed in table 4.2 were within normal ranges except for ESR. At visit A, ESR had a median of 11.5 mm/hr (IQR: 7.75 – 16.5 mm/hr) and at visit B, a median of 26 mm/hr (IQR: 15.75

– 39.25 mm/hr). These readings were higher than those compared to ranges in healthy children which ranged between 0 -10 mm/hr (NHLS, TrakCare Database).

Table 4.2: Descriptive statistics for participant serum calprotectin (S100A8/A9) and routine laboratory findings at visit A and visit B.

	Visit A Median (IQR)	**Visit B** Median (IQR)	**p-value**
Calprotectin (ng/mL)	628.6 (422.85 - 918.95)	1069.1 (511.8 - 1379.5)	0.3394
CRP (mg/mL)	1 (0.9 - 1.75)	1 (1 – 5)	0.1328
ESR (mm/hr)	11.5 (7.75 - 16.5)	26 (15.75 - 39.25)	**0.0068**
RCC (x 10^{12}/L)	4.65 (4.36 - 4.95)	4.77 (4.49 - 4.91)	0.9102
Haemoglobin (g/dL)	12.60 (11.60 - 13.30)	12.75 (11.90 - 13.33)	0.5347
Platelet Count (x 10^9/L)	314 (286 - 350)	315.5 (295 - 355.25)	0.3911
WCC (x 10^9/L)	6.93 (6.18 - 8.33)	7.23 (5.96 - 8.83)	0.1514
Neutrophils (x 10^9/L)	4 (2.98 - 4.73)	4.53 (3.28 - 5.76)	0.2334
Lymphocytes (x 10^9/L)	2.17 (1.79 - 2.72)	2.04 (1.90 - 2.65)	0.9453
Monocytes (x 10^9/L)	0.39 (0.27 - 0.45)	0.34 (0.21 - 0.46)	0.3672
Eosinophils (x 10^9/L)	0.12 (0.10 - 0.24)	0.13 (0.11 - 0.24)	0.2593
Basophils (x 10^9/L)	0.04 (0.02 - 0.05)	0.06 (0.04 - 0.07)	**0.0273**

RCC: Red Cell Count, WCC: White Cell Count

Normal ranges (R&D Systems® Inc. 2015), in children, for calprotectin and routine laboratory findings ((NHLS TrakCare database)) are listed below:

Calprotectin (S100A8/A9)	127	-	1395	ng/mL
CRP		<	10	mg/mL
ESR	0	-	10	mm/hr
Red Cell Count	3.8	-	4.8	x 10^{12}/L
Haemoglobin	12	-	15	g/dL
Platelet Count	186	-	545	x 10^9/L
White Cell Count	3.9	-	12.6	x 10^9/L
Neutrophils	1.6	-	8.3	x 10^9/L
Lymphocytes	1.4	-	4.5	x 10^9/L
Monocytes	0.2	-	0.8	x 10^9/L
Eosinophils	0	-	0.4	x 10^9/L
Basophils	0	-	0.1	x 10^9/L

Table 4.3 lists the descriptive statistics for the 9 additional inflammatory biomarkers of interest in this study, at visit A and B. There were minimal changes to inflammatory marker expression from visit A to B.

Table 4.3: Descriptive statistics for additional inflammatory biomarkers investigated in this study for visit A and visit B.

	Visit A Median (IQR)	**Visit B** Median (IQR)	**p -value**
sCD14 x 10^3 (pg/mL)	1299.6 (1043.7 – 1524.9)	1306 (1221.5 – 1700)	0.3394
CD163 x 10^3 (pg/mL)	256.4 (230.9 - 329.1)	341.8 (286.6 - 537.2)	0.2439
MCP-1 (CCL2) (pg/mL)	186.3 (118.9 - 239.2)	141.9 (113.1 - 255.0)	0.7354
Eotaxin (CCL11) (pg/mL)	107.1 (82.0 - 136.8)	107.0 (65.4 - 153.6)	0.5781
TNF-α (pg/mL)	4.4 (3.0 - 6.1)	4.4 (3.6 - 5.8)	0.4248
MDC (CCL22) (pg/mL)	843.5 (620.2 - 1210.6)	958.8 (613.4 - 1312.0)	0.6355
IL-2Rα (CD25) (pg/mL)	593.5 (480.2 - 810.7)	819.5 (627.0 - 887.4)	0.6848
IP-10 (CXCL10) (pg/mL)	34.6 (20.5 - 43.9)	40.6 (32.5 - 43.8)	0.9460
MIF (pg/mL)	1129.1 (733.0 - 1581.1)	946.1 (492.0 - 1248.4)	>0.9999

Table 4.4 summarises the monocyte-related findings. These include total monocytes for the cohort at visit A and B, as well as the percentage of each monocyte surface marker and the distribution of monocyte subsets of all collected samples at both visits.

Table 4.4: Descriptive statistics for total monocytes, surface marker expression and monocyte subset distribution at visit A and visit B.

	Visit A Median (IQR)	**Visit B** Median (IQR)	**p -value**
Number of participants	22	13	
Total monocytes (% total cells)	4.8 (4.2 – 6.1)	4.5 (3.6 – 5.3)	0.0503
Monocyte surface markers (% expression on total monocytes)			
CD86	68.4 (2.4 - 85.9)	86.7 (81.9 - 91.1)	0.0942
CD36	86.1 (62.4 - 91.6)	87.9 (80.8 - 92.7)	0.4973
CD64	76.0 (59.7 - 84.7)	83.8 (76.7 - 85.8)	0.1968
TLR4	0.8 (0.4 - 2.9)	1.1 (0.7 - 1.5)	0.5759
Monocyte Subsets: (% total monocytes)			
Classical	40.2 (2 - 62.88)	23.1 (13.1 - 52.6)	0.3054
Intermediate	27 (10.36 - 41.78)	39.6 (22.5 - 56.4)	0.3054
Non-classical	9.5 (5.53 - 16.13)	10.3 (6.93 - 11.1)	0.7354

At visit A, non-classical monocytes (Median: 9.5%; IQR: 5.5-16.1%) were within the normal range of 2 - 10%, classical monocytes (Median: 40.2%; IQR: 2-62.9) were lower than the normal range of 80-90%, and intermediates (Median: 27%; IQR: 10.4-41.8%) were higher than the normal range of 2-5%. Similarly at visit B, non-classical monocytes were within the normal range (Median: 10.3%; IQR: 6.9-11.1%), intermediates were higher than normal (median: 39.6%; IQR: 22.5 - 56.4%), and classicals were lower than the normal range (median: 23.1%; IQR: 13.1 - 52.6).

In relation to monocyte surface marker expression at visit A, CD86 (Median: 68.4%; IQR: 1.5 – 86.3%) was higher than the expected ranges of 5.3% ± 1.4% in females, or 5.4% ± 1.6% in males (Martínez-García et al. 2020). CD64 expression (Median: 76%; IQR: 58.1 – 85.3%) was also higher than the expected range of between 14.3 – 53.9% (Karawaiczyk et al. 2021). In contrast, CD36 expression (Median: 86.1%; IQR: 62.2 – 92.4%) was lower than the expected amount of >95% of total monocytes (Moniuszko et al. 2006) and Expression of TLR4 (Median: 0.82%; IQR: 0.4 – 3.3%) was also much lower than the expected ranges of either 14.1 ± 10.4 in females or 8.9 ± 9.7% in males (Martínez-García et al. 2020).

Similarly, at visit B, CD86 (Median: 86.7%; IQR: 81.9 - 91.1%) and CD64 (Median: 83.8%; IQR: 76.7 - 85.8%) expression was higher than expected, while CD36 (Median: 87.9%; IQR: 80.8 - 92.7%) and TLR4 expression (Median: 1.1%; IQR: 0.7 - 1.5%) was lower than expected ranges.

4.2.1. Sample Stratification for Analysis

Using all participant samples collected at visit A and B, specific grouping and stratification was done to investigate and identify any potential differentiators of disease status or inflammation, as well as how calprotectin would relate to these stratified groups (Figure 4.1). Samples were grouped based on a matched sample strategy i.e., only those who would return later for visit B (n=13), by disease phenotype (active disease or in remission) and stratified groups based on ESR (high or low ESR).

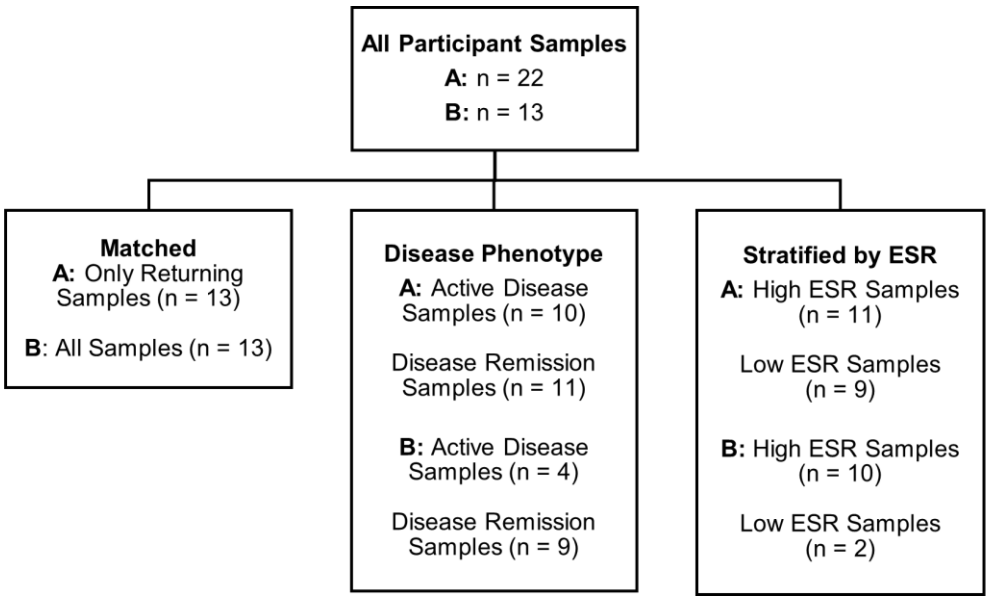


Figure 4.1. Flow chart showing the grouped samples for additional analysis at visit A and B. Of all samples collected, returning participant samples were grouped for analysis, disease course i.e., active disease or remission were grouped for analysis and measurements of routinely assessed ESR i.e., high, and low ESR samples were grouped for analysis. Samples with no information relating to the stratifying variables were excluded from the analysis.

Summary statistics for the ESR stratified and disease phenotype groups are tabulated in Appendix I and Appendix H, respectively.

4.3 Calprotectin and Routine Markers of Inflammation

The following correlation analysis was performed on all analysis groups at visit A and B. The results below describe the relationships at visit A only, and additional results relating to visit B have been included in Appendix F.

Median expression of calprotectin in all samples at baseline was 628.6 ng/mL (IQR: 406.2-979.8 ng/mL). Figure 4.2A, B and C illustrate the relationships between the main markers of inflammation, that is, calprotectin (S100A8/A9), CRP and ESR. There were significantly positive correlations between calprotectin and CRP (p= 0.0063; r= 0.5638) and CRP and ESR (p= 0.0032; r= 0.6253). There was, however, no significant correlation between calprotectin and ESR (p= 0.1400; r= 0.3419).

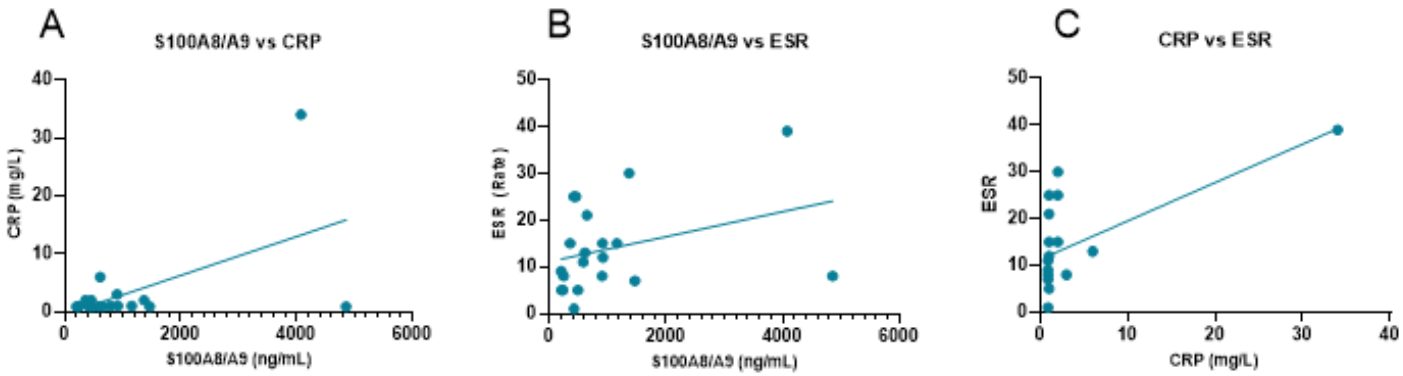


Figure 4.2. Scatter plots showing correlations between routine markers of inflammation and S100A8/A9 for all participants at visit A. A. Calprotectin (S100A/A9) correlated with CRP (p= 0.0063; r= 0.5638); B. Calprotectin did not significantly correlate with ESR (p= 0.1400; r= 0.3419); C. CRP correlated with ESR (p= 0.0032; r= 0.6253). The straight line is a linear regression line to represent the relationship between variables.

Only 3 samples at visit A had higher than normal calprotectin expression (> 1395 ng/mL) i.e., participant 6, 10 and 20.

Returning participants (matched analysis group)

Data for the 13 participants only at visit A, that would return for routine follow ups at visit B, were analysed. Only 2 participants samples (15.4%) had higher than normal calprotectin expression (> 1395 ng/mL) in this group i.e., participant 10 and 20.

Median expression of calprotectin in this matched analysis group was 646 ng/mL (IQR: 458.4-1263). Figure 4.3A, B and C illustrate the relationships between calprotectin (S100A8/A9) and the routine markers of inflammation, CRP and ESR, within this analysis group. There were significantly positive correlations between calprotectin and CRP (p< 0.0001; r= 0.9166), CRP and ESR (p= 0.0197; r= 0.6594) and calprotectin and ESR (p= 0.0129; r= 0.6907).

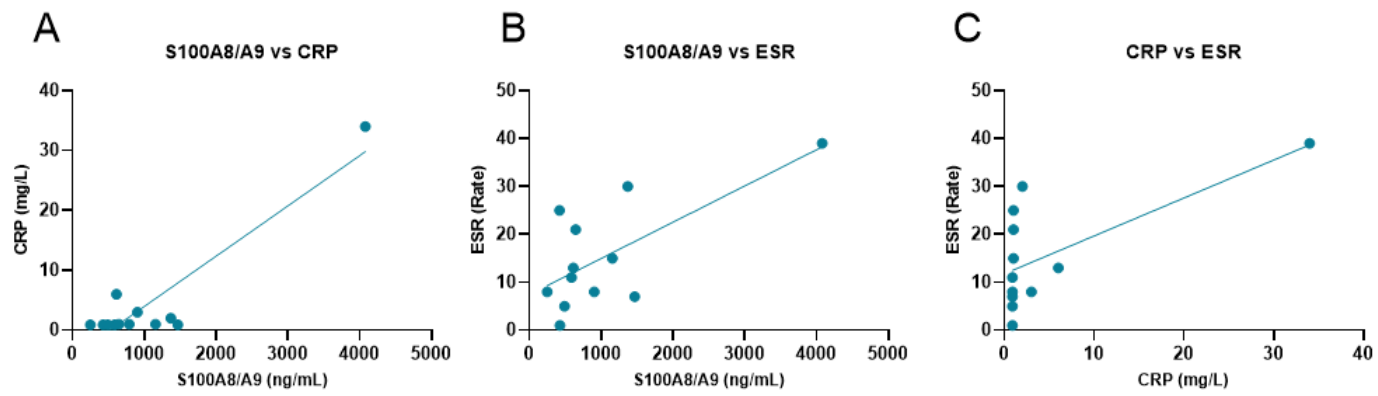


Figure 4.3. Scatter plots showing correlations between routine markers of inflammation and S100A8/A9 at baseline for paired (n = 13) samples. A. Calprotectin (S100A8/A9) correlated with CRP (p<0.0001; r=0.9166); B. Calprotectin (S100A8/A9) correlated with ESR (p=0.0129; r=0.6907); C. CRP correlated with ESR (p=0.0197; r=0.6594).

Stratified by disease phenotype (active and remission groups)

Only 2 participants samples (18.2%) expressed higher than normal calprotectin in the active group i.e., participants 6 and 20, while only 1 participant (10%) in the remission group i.e., participant 10, expressed abnormally high calprotectin (> 1395 ng/mL).

Median expression of calprotectin in the active and remission groups were 654.1 ng/mL (IQR: 384-876) and 611.2 ng/mL (IQR: 458.3-1038.8), respectively. The relationships between calprotectin and routine inflammatory markers (CRP & ESR) for the active disease group are illustrated in Figure 4.4A, B and C. There were no statistically significant relationships between calprotectin and CRP (p= 0.6108; r= -0.1731), calprotectin and ESR (p= 0.5868; r= -0.2104) or CRP and ESR (p= 0.4374; r= 0.2972).

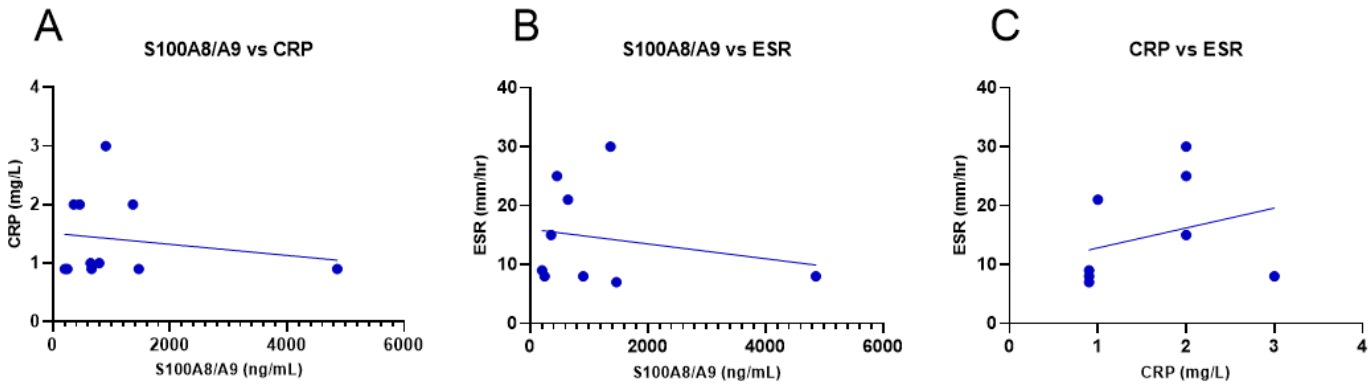


Figure 4.4: Scatter plots showing correlations between routine markers of inflammation and S100A8/A9 at baseline for the active disease group. A. Calprotectin (S100A8/A9) did not significantly correlate with CRP (p=0.6108; r=-0.1731); B. Calprotectin (S100A8/A9) did not significantly correlate with ESR (p=0.5868; r=-0.2104); C. CRP did not significantly correlate with ESR (p=0.4374; r=0.2972).

The relationships between calprotectin and routine inflammatory markers (CRP & ESR) for the disease remission group are illustrated in Figure 4.5A, B and C. There were significantly positive correlations between calprotectin and CRP (p< 0.0001; r= 0.9555), CRP and ESR (p= 0.0060; r= 0.7944) and calprotectin and ESR (p= 0.0036; r= 0.8215).

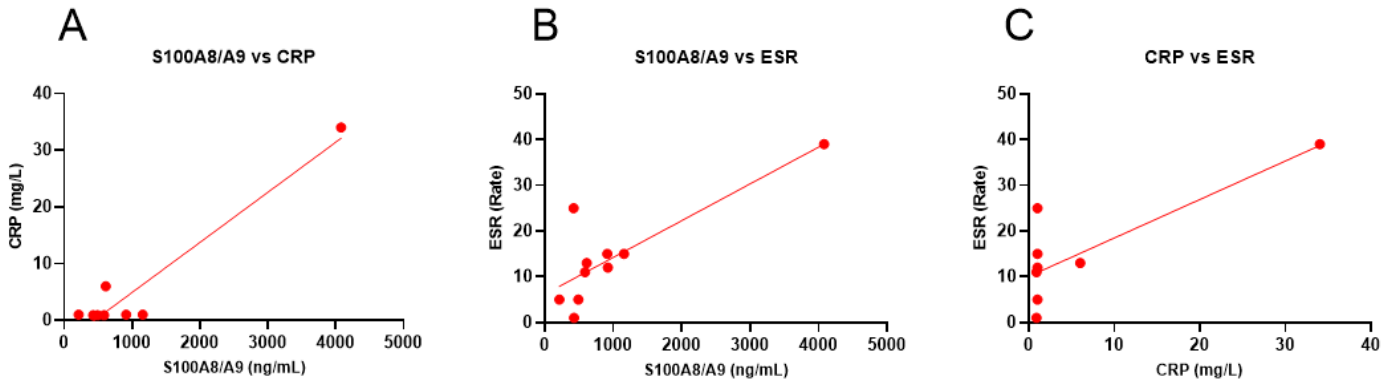


Figure 4.5: Scatter plots showing correlations between routine markers of inflammation and S100A8/A9 at baseline for the disease remission group. A. Calprotectin (S100A8/A9) correlated with CRP (p<0.0001; r= 0.9555); B. Calprotectin (S100A8/A9) correlated with ESR (p=0.004; r=0.6205); C. CRP correlated with ESR (p=0.0202; r=0.4683). The straight line is a linear regression line to represent the relationship between variables.

Stratified according to ESR measurements (high vs low ESR)

Median expression of calprotectin in the high and low ESR analysis groups were 646 ng/mL (IQR: 456.7-1157) and 426.3 ng/mL (IQR: 223.4-1186), respectively. The relationships between calprotectin and routine inflammatory markers (CRP & ESR) for the high ESR group are illustrated in Figure 4.6A, B and C. There were significantly positive correlations between calprotectin and CRP (p< 0.001; r= 0.9361), CRP and ESR (p= 0.0187; r= 0.6903) and calprotectin and ESR (p= 0.0145; r= 0.7095).

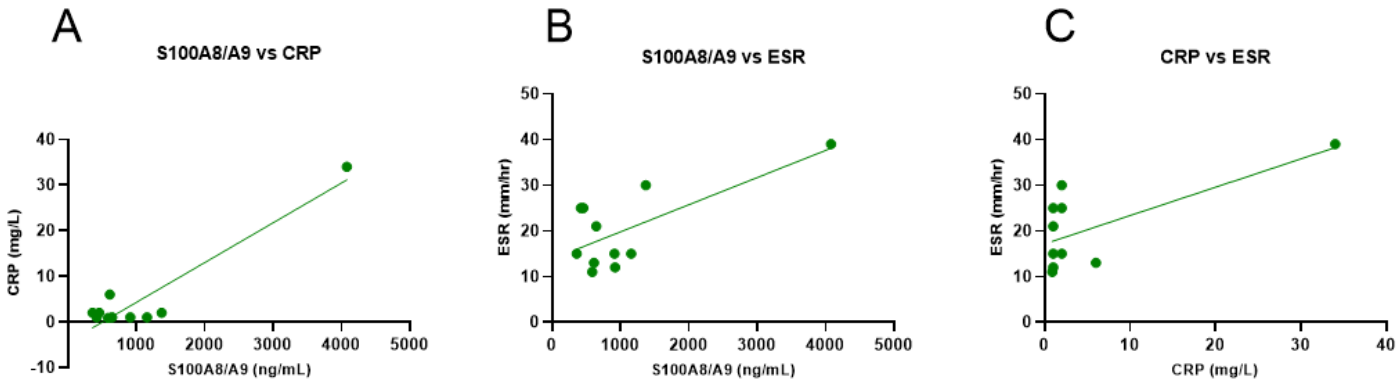


Figure 4.6: Scatter plots showing correlations between routine markers of inflammation and S100A8/A9 at baseline for the stratified group with a high ESR. A. Calprotectin (S100A8/A9) correlated with CRP (p<0.001; r=0.9361); B. Calprotectin (S100A8/A9) correlated with ESR (p=0.0145; r=0.7095); C.

CRP correlated with ESR (p=0.0187; r=0.6903). The straight line is a linear regression line to represent the relationship between variables.

The relationships between calprotectin and routine inflammatory markers (CRP & ESR) for the low ESR group are illustrated in Figure 4.7A, B and C. There were no statistically significant correlations between calprotectin and CRP (p= 0.9294; r= -0.0347), CRP and ESR (p= 0.4987; r= 0.2603) or calprotectin and ESR (p= 0.4310; r= 0.3012).

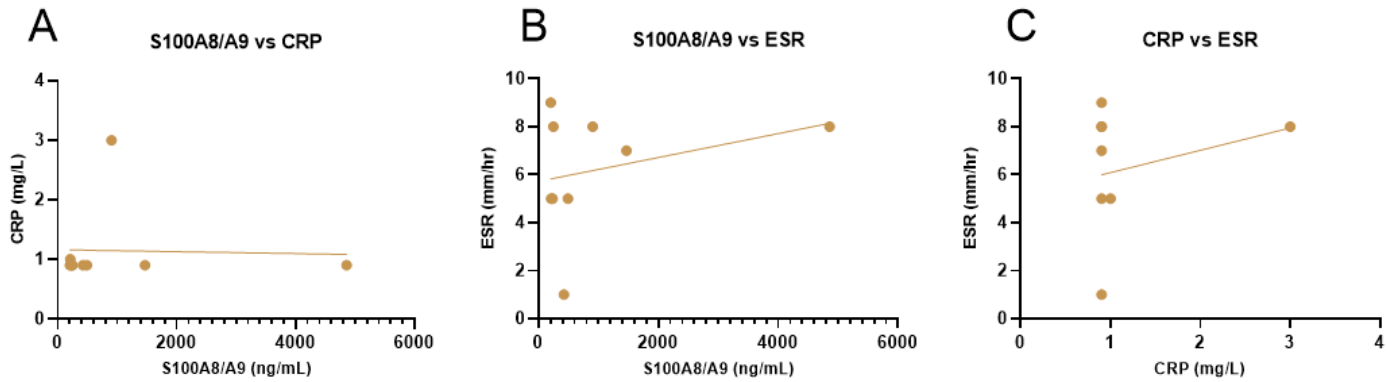


Figure 4.7. Scatter plots showing correlations between routine markers of inflammation and S100A8/A9 at baseline for the stratified group with low ESR. A. Calprotectin (S100A8/A9) did not correlate with CRP (p=0.9294; r=-0.0347); B. Calprotectin (S100A8/A9) did not correlate with ESR (p=0.4310; r=0.3012); C. CRP did not correlate with ESR (p=0.4987; r=0.2603). The straight line is a linear regression line to represent the relationship between variables.

Figure 4.8 shows that calprotectin (p=0.3394) and CRP (p=0.1328) did not change significantly between visits. Similarly, in all other analysis groups, calprotectin and CRP did not change significantly over time.

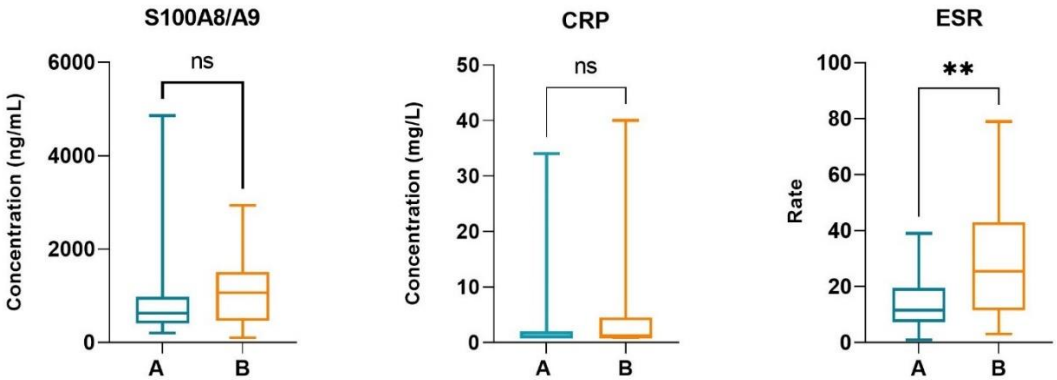


Figure 4.8. Box-and-whisker plots showing the changes in expression of the main markers of inflammation for all participants over time, from visit A to B. No significant change (p=0.3394) was seen for calprotectin (S100A8/A9) or CRP (p=0.1328). The increase in ESR was significant (p=0.0068).

CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate

However, ESR data shows that there was a statistically significant (p=0.0068) increase, from visit A to visit B, within all participant samples. This was not seen among the other analysis groups, i.e., disease phenotype or ESR stratified groups.

4.4 Calprotectin and Inflammatory Cytokines

Upon analysis of the correlation matrix of all samples collected at visit A and visit B, to observe any significant relationships between calprotectin and the inflammatory markers included in our investigations, no significant relationships were identified. A trend toward significance was observed between calprotectin and IP-10 at baseline (p= 0062; r= 0.404). This trend was also observed in the low ESR group at visit A only (p= 0.0756; r= 0.6189) and the active disease group at visit A only (p= 0.064; r= 0.576). No further significant relationships were identified between calprotectin and the inflammatory biomarkers when assessing the remaining analysis groups illustrated in Figure 4.1 at visit A or visit B.

Figure 4.9 below shows the changes in biomarker expression between visit A and B. There were no significant trends when evaluating these markers over time across all analysis groups. Extreme outliers (> 1.5 x Q3) of MIF were excluded from the data. Concentrations of sCD14 and CD163 were scaled down by a factor of 10^{-3} to make the data more comparable in this format.

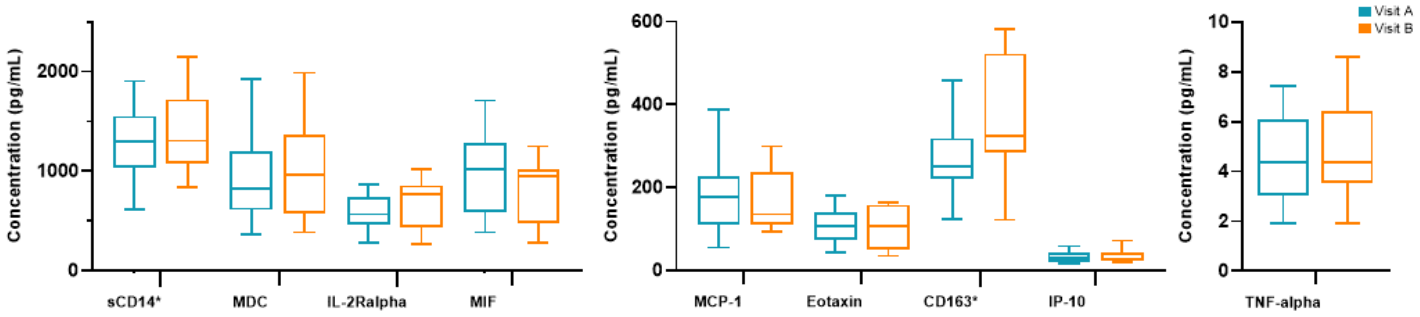


Figure 4. 9. Box-and-whisker plot showing the concentrations of biomarkers in all samples from visit A to B. No significant changes in biomarker expression were observed. Markers were grouped and graphed based on the magnitude of expression to better interpret the data. The rectangular box represents the IQR with the line in the middle representing the median. Extreme outliers (>1.5 x Q3) were excluded. *Concentrations of sCD14 and CD163 were scaled down by a factor of $x10^{-3}$ to make data more comparable in this format.
CD: Cluster of Differentiation, MCP: Monocyte Chemoattractant Protein, TNF: Tumour Necrosis Factor, MDC: Macrophage-derived Chemokine, IL: Interleukin, IP: Interferon-gamma-induced protein, MIF: Macrophage Migration Inhibitory Factor

4.5 Calprotectin and Monocytes

Calprotectin did not correlate significantly to the monocyte subsets in any of the analysis groups, except for the active disease group at visit B which showed a significant correlation between calprotectin and the intermediate monocyte subset (p= 0.0187; r= -0.9813). Within this analysis group at visit B, the intermediate monocytes also correlated significantly with CRP (p=0.0346; r=-0.9654).

In relation to the routine markers of inflammation, the intermediate monocyte subset also correlated significantly with ESR (p=0.0391; r=0.4427) and CRP (p=0.0021; r=0.6451) in all samples at visit A. Similarly, intermediate monocytes correlated with ESR (p=0.0162; r=0.6740) in the matched group at visit A.

The same was true for the disease remission group at visit A, with intermediate monocytes correlating significantly with ESR (p=0.0314; r=0.6773) and CRP (p=0.0056; r=0.7990). The high ESR group showed a significantly positive relationship between the intermediate monocytes and ESR (p=0.0321; r=0.6452) and no significant relationships were identified in the low ESR group at either visit.

The distribution of monocyte subsets, within all samples at visit A and B, are illustrated in Figure 4.10 below. Total monocytes and monocyte subsets (as a percentage of total monocytes) did not change significantly in all participant samples and analysis groups, from visit A to B.

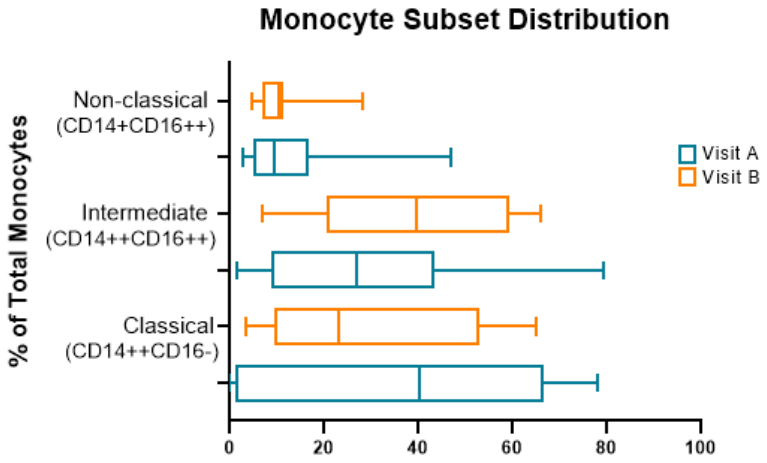


Figure 4 10. Box-and-whisker plot showing monocyte subset distribution of all samples collected at visit A and visit B. The rectangular box represents the IQR with the line in the middle representing the median. There were no statistically significant differences from visit A to B for any of the monocyte subsets.

The normal ranges for these subsets were:

Classical Monocytes (CD14++CD16+)	80	-	90	%
Intermediate Monocytes (CD14++CD16++)	2	-	5	%
Non-classical Monocytes (CD14+CD16++)	2	-	10	%

No significant correlations were identified between calprotectin and the monocyte surface markers. Expression of monocyte surface markers CD36, CD64, CD86 and TLR4 were investigated on all monocytes and monocyte subsets (Appendix G). There were no significant changes in CD36, CD64 or TLR4 expression over time for all participants (Figure 4.11), and within the other analysis groups, except for the remission group (Figure 4.12).

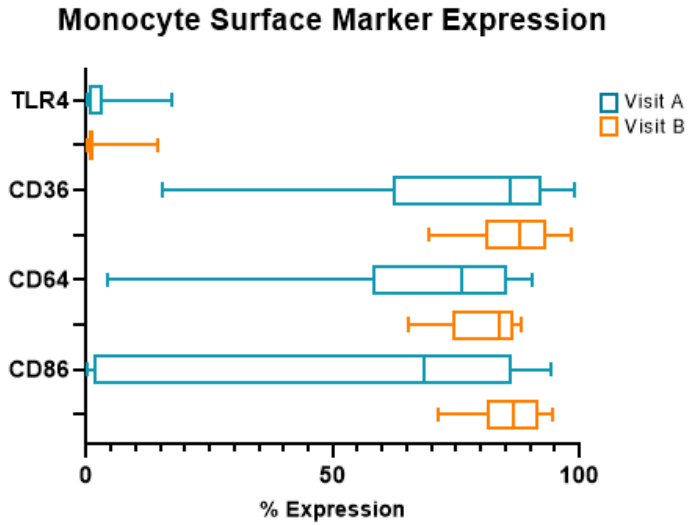


Figure 4.11. Box-and-whisker plot showing percentage surface marker expression of CD36, CD64, CD86 and TLR4 on total monocytes of all participant samples at visit A and visit B. The rectangular box represents the IQR with the line in the middle representing the median.

Ranges in healthy controls:

CD86 Expression	5.3	±	1.4 (mean ± SD)	% for women	
	5.4	±	1.6 (mean ± SD)	% for men	(Martínez-García et al. 2020)
CD64 Expression	14.3	-	53.9	%	(Karawaiczyk et al. 2021)
CD36 Expression		>	95	%	(Moniuszko et al. 2006)
TLR4 Expression	14.1	±	10.4 (mean ± SD)	% for women	
	8.9	±	9.7 (mean ± SD)	% for men	(Martínez-García et al. 2020)

Figure 4.12 below illustrates the changes in monocyte marker expression over time in the remission analysis group. The only significantly different finding was that for CD86 expression, which increased ($p= 0.0273$) from visit A to visit B.

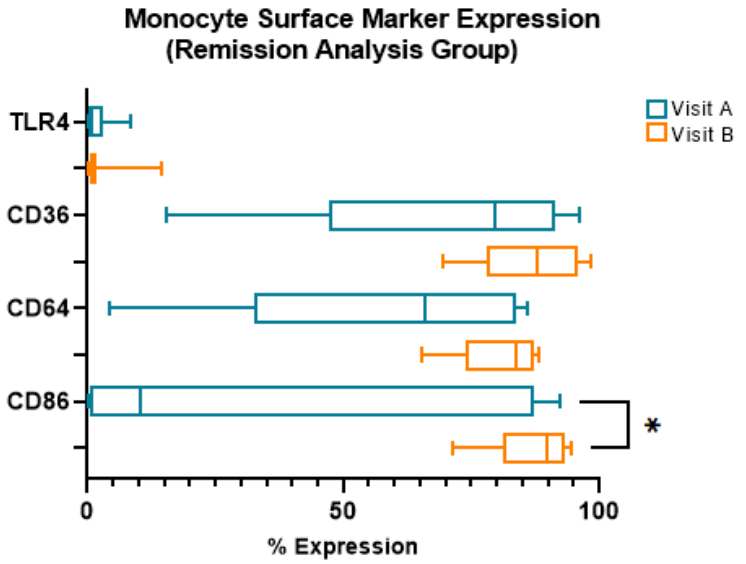


Figure 4. 12. Box-and-whisker plot showing the changes in monocyte surface marker expression over time. CD86 expression increased significantly (p=0.0273) from visit A (n=10) to visit B (n=9) in the remission group.

None of the other analysis groups showed evidence for significant changes in monocyte surface marker expression over time.

4.6 Calprotectin and Disease Activity

Relationships between calprotectin and disease activity, measured by the number of active joints, are described below.

Calprotectin did not correlate significantly with the number of active joints in all patient samples at visit A, however, at visit B the correlation was significant (p=0.0444; r=0.588). The number of active joints did however correlate significantly with IP-10 (p=0.001; r=0.679) at visit A and showed a trend toward significance with MDC (p=0.051; r=-0.582) at visit B. Within the matched group, calprotectin did not correlate with the number of active joints.

Calprotectin correlated significantly with the number of active joints (p=0.027; r=0.973) and CRP (p=0.039; r=0.780) in the active disease group at visit B, within this group at visit B, the number of active joints also correlated significantly with the percentage of intermediate (p=0.010; r=-0.990) and classical monocytes (p=0.024; r=0.976). A significant correlation between active joints and IP-10 (p=0.045; r=0.643) in the active disease group at visit A was also identified. Calprotectin did not correlate significantly in the remission groups at either visit, however, the number of active joints did correlate with CD163 (p=0.026; r=0.694), IL-2Rα (p=0.003; r=0.837) and MIF (p=0.024; r=0.700), and TLR4 expression on monocytes (p=0.002; r=0.855) with a trend toward significance with MDC (p=0.054; r=0.624). While calprotectin did not correlate significantly with active joint count at visit B for the remission group, active joints did have a significantly positive relationship with MCP-1

(p=0.008; r=0.844), MIF (p=0.002; r=0.901) and a trend toward significance with MDC (p=0.052; r=0.703) and IL-2Rα (p=0.070; r=0.668).

Calprotectin and number of active joints did not correlate significantly in the high or low ESR groups at both visits.

No statistically significant differences in active joint counts were identified from visit A to visit B among any of the analysis groups.

4.7 Case Studies

Cases with elevated calprotectin or compliance issues, are tabulated for visit A and B below to investigate the implications of elevated calprotectin and poor compliance on disease activity and risk of relapse. The table also includes information relating to participant 16, which was the only treatment naïve participant recruited at visit A. Clinical information is detailed in Table 4.5 and laboratory findings are detailed in Table 4.6.

Table 4. 5: Case study participant clinical information for baseline and follow-up.

Patient ID	Age	Sex	Diagnosis	Sample collection date	Clinical presentation	Disease Activity (Active joint count)	Current treatment regimen	Time on Treatment	Compliant (Y/N)	Disease flare after visit (Y/N)	Notes
10 A	15	F	Oligoarticular JIA; ANA (+)	06-Oct-20	JAS, learning difficulties, no complaints, erythematous rash over L molar and under eyes	Inactive (0)	MTX, HCQ, Enbrel, VitD, INH	2015-present	N	N	Poor compliance
10 B				02-Mar-21	No complaints – clinical remission	Inactive (0)	MTX, HCQ, Enbrel, VitD, INH		N	Y (relapse)	
16 A	2	F	New onset Oligoarticular JIA; ANA +; Bilateral Uveitis	13-Oct-20	L – ankle swollen, tender, limping	Moderate (1)	Brufen, Panado	Naïve patient	Y	N	Next step is weaning medication
16 B				30-Mar-21	R – eye inflamed cells Stable remission	Inactive (0)	MTX, FA, Brufen, Ca, VitD, Zn, Prednisone (wean steroids & add biologics, wean pred)		Y	N	
20 A	14	F	Polyarticular JIA (RF -)	27-Oct-20	Fingers painful & stiff in the morning, wrists are tender, poor compliance	Moderate (12)	MTX, FA, Sulfasalazine	2019-present	N	Y	Transport issue for medications
20 B				01-Jun-21	No complaints except wrists, apparently compliant		MTX, FA, Sulfasalazine, HCQ, VitD, Zn, Mtv		N	Y	
21 A	16	M	sJIA	27-Oct-20	Wrist, elbow & hip pain; compliance issue	Moderate (8)	MTX, FA, Prednisone, HCQ, VitD, Zn, Mtv	2017-present	N	Y	Supervision a problem wrt medications
21 B				01-Jun-21	Wrists, and knee limited ROM &	Moderate (8)	MTX, FA, HCQ, VitD, Zn, Mtv, PenVK		N	Y	

					tenderness, compliance issues						Cousin recently admitted with sJIA as well
22 A	18	F	Polyarticular JIA (RF -)	27-Oct-20	R - hand, jaw painful	Low (3)	MTX, FA, Tocilizumab, HCQ, Prednisone, Mtv	2016-present	Y	N	Failed 4 biologics so far
22 B				13-Apr-21	Fell last week - painful hip and back area; R - hand stiff & painful, MCP limitations.	Moderate (2)	MTX, FA, Tocilizumab, HCQ, Prednisone, VitD, Ca, Mtv		Y	Y	

R – Right, L – Left, MTX: Methotrexate, FA: Folic Acid, VitD: Vitamin D, PenVK: Penicillin, HCQ: Hydroxychloroquine, Mtv: Multivitamin, Zn: Zinc, Ca: Calcium, VitC: Vitamin C, INH: Isoniazid

Information provided on disease flares after visit B and notes were recorded during participant review with Dr Deepthi Abrahams.

Table 4. 6: Inflammatory biomarker expression and monocyte distribution at Visit A and B for participant case studies.

Patient ID	Calprotectin (S100 A8/A9) ng/mL	CRP mg/mL	ESR mm/hr	sCD14 pg/ml	MCP-1 pg/ml	Eotaxin pg/ml	CD163 pg/ml	TNFα pg/ml	MDC pg/ml	IL-2Rα pg/ml	IP-10 pg/ml	MIF pg/ml	Monocytes x 10^9/L	Classical	Intermediate	Non-classical
10 A	4079.8 H	34 H	39 H	1329400	90.83	44.64	300260.71	6.21	724.72	744.75	30.61	1709.2	0.49	1.45	78.4	17.4
10 B	2879.3 H	40 H	59 H	1305700	141.93	72.36	481667.09	8.02	1312.03	832.5	71.7	1007.67	0.43	23.1	41.7	7.78
16 A	791.5	1	NS	1687600	242.66	130.55	386672.76	7.42	1921.92	1538.25	37.06	4648.38	0.43	52.2	28.70	12
16 B	101.4 L	5	NS	1768700	254.98	35.38	1048000	8.62	1223.41	1508.15	71.06	2177.51	OOR	30.8	53.7	11.1
20 A	1467.1 H	<1	7	1013600	305.3	93.74	255059.8	3.32	1061.1	473.44	149.35	724.94	0.42	50.3	25.30	11.1
20 B	1630.5 H	3	24 H	892033.38	299.42	150.99	283840.87	3.94	958.75	438.75	142.71	946.09	0.54	3.54	64.4	28.1
21 A	904.2	3	8	1145600	97.91	OOR	227882.06	5.76	619.95	798.89	32.05	757.14	0.32	78.1	1.56	2.91
21 B	2937.7 H	28 H	30 H	1267100	125.6	OOR	308589.49	3.48	524.74	819.49	21.81	946.09	0.49	52.6	32.3	10.3
22 A	248.5	<1	8	615572.9	115.81	OOR	588244.6	3.02	405.74	1284.95	74.02	492.02	0.20	41.1	30.20	15
22 B	1379.5 H	1	36 H	838226.82	108.56	OOR	560677.73	2.4	387.32	856.39	43.54	946.09	0.21	20.2	56.4	11

OOR – Out Of Range (below or above detection threshold)

Normal ranges, in children, for main inflammatory markers & monocyte findings are listed below:

Calprotectin (S100A8/A9)	127	-	1395	ng/mL	(R&D Systems® Inc. 2015)
CRP		<	10	mg/mL	
ESR	0	-	10	mm/hr	
Monocytes	0.2	-	0.8	x 10^9/L	(NHLS TrakCare database)
Classical Monocytes (CD14++CD16+)	80	-	90	%	
Intermediate Monocytes (CD14++CD16++)	2	-	5	%	
Non-classical Monocytes (CD14+CD16++)	2	-	10	%	

Participant 10

At visit A, disease activity was 'inactive' with no active joints recorded. Similarly, no complaints or active joints were recorded at visit B and participant was in clinical remission on treatment. This participant was not fully compliant on treatment (Table 4.5). Expression of calprotectin, CRP and ESR were higher than normal ranges in children. Increases in plasma expression of MCP-1, eotaxin, CD163, MDC, IL-2Rα and IP-10 can be seen at visit B. The disease flare after visit B indicated relapse of disease. Classical monocytes increased in conjunction with a decrease in intermediate and non-classical monocytes (Table 4.6).

Participant 16

At visit A, this participant was treatment naïve and had not yet been on any anti-rheumatic treatment. One active joint was recorded at visit A. Following treatment, no active joints were recorded at visit B and the participant was in stable remission on treatment (Table 4.5). Calprotectin was normal at visit A but decreased to extremely low levels following initiation of treatment. Decreases in eotaxin, MDC and MIF were also observed alongside an increase in intermediate monocytes. No disease flare was recorded after visit B (Table 4.6).

Participant 20

At visit A, disease activity was moderately active with 12 active joints recorded. Poor compliance on treatment was recorded throughout the study for this participant. Painful and stiff wrists were recorded at visit B (Table 4.5). Calprotectin remained higher than normal at both visits, and ESR increased to above normal at visit B. CRP remained stable throughout. Additionally, monocyte subset distribution revealed a decrease in classical monocytes and an increase in intermediate and non-classical monocytes at visit B. Increases in ESR, eotaxin and MIF expression was recorded from visit A to B (Table 4.6).

Participant 21

Moderate disease activity was recorded with involvement of 8 active joints at both visit A and B. The participant was poorly compliant on medication and experienced disease flares after both visits (Table 4.5). Calprotectin, CRP and ESR were stable at visit A and then increased to above normal levels at visit B. Additionally, sCD14, MCP-1, CD163, IL-2Rα and MIF increased from visit A to B. Furthermore, classical monocyte numbers decreased alongside an increase in intermediate and non-classical monocytes (Table 4.6).

Participant 22

Participant 22 has an aggressive JIA history with failure of 4 biologic treatments. Active joints were recorded at both visits and a disease flare was confirmed after visit B. Compliance on treatment was

not an issue (Table 4.5). Expression of plasma calprotectin and ESR increased above the normal range at visit B. Additionally, increases were observed in expression of sCD14 and MIF, as well as numbers of intermediate monocytes (Table 4.6).

Chapter 5 Discussion

This chapter consists of 4 sections. The first (5.1) discusses the participants, their clinically relevant information and routine laboratory results. The second (5.2) looks at the distribution of monocyte subsets and marker expression in relation to JIA inflammation. The third (5.3) discusses significant findings related to the inflammatory biomarkers involved in JIA, including calprotectin, which is the focus of this study. The fourth (5.4) discusses unique case studies and the final (5.5) relates to the overall findings and limitations.

The original study design included assessment of treatment naïve JIA patients, as well as those on treatment, over time to observe the changes in calprotectin as a marker for treatment monitoring. Minimal patients were treatment naïve at the time of recruitment and the final cohort included majority JIA patients already on treatment. These patients were followed over time and the focus was then shifted to stratifying the data based on disease phenotypes (active and remission) and ESR (high and low ESR) to identify any potential markers of disease activity or inflammatory activity within these groups.

5.1 Participants

Phenotypic grouping, that is, into active disease and disease remission, was done to identify potential biomarkers of disease status. Similarly, total collected samples could show any trends present in our cohort and therefore linked to the areas surrounding Tygerberg, however, such findings are hindered by the small sample size. The small sample size is reflective of the low prevalence of the disease and/or a lack of referral to a tertiary centre. Originally, 20-30 participants were to be recruited to the study and followed up at both 3- and 6-months. Currently, there were 22 participants recruited of which, only 13 returned after 6 months for follow ups.

The major confounding factor affecting the original study design was that all patients were undergoing ant-inflammatory treatment (except Participant 16). Expression of ESR however, still showed the most evidence for changes in inflammation compared to the other acute phase reactant, CRP, which was within the normal range for almost all participant samples. Therefore, stratifying for ESR allowed us to identify possible markers of apparent inflammation, even on anti-inflammatory treatment regimes. Kotulska et al. confirmed the usefulness of ESR in monitoring disease activity of JIA (Kotulsa et al. 2015).

5.1.1. Clinical information

The most prevalent subtypes of JIA among the study participants were oligoarticular JIA and polyarticular RF negative JIA, which was consistent with findings in another JIA study conducted in the Western Cape in 2012 (Weakley et al. 2012). Al-Mayouf et al. also identified oligoarticular as the most prevalent subtype, followed by polyarticular RF negative JIA and sJIA, in Africa and the Middle East, based on a review of existing literature (Al-Mayouf et al. 2021). In contrast however, another study which evaluated prevalence at a sub-Saharan level identified oligoarticular JIA to be the least prevalent in this region; It was suggested that this lack of oligoarticular cases was due to underdiagnosis and delayed referral in this setting which has a major lack of funding and expertise surrounding paediatric rheumatology (Scott and Webb 2014). Given the small sample size used for this study, no definitive comments can be made regarding subtype prevalence in the Western Cape as a whole.

The mean age at recruitment was 13.2 years. The current age of patients attending Tygerberg's paediatric rheumatology clinic was higher than that seen in studies conducted in Europe, Turkey, and Morocco (Weakley et al. 2012). The older age could be attributed to delayed referral to tertiary treatment centres due to the socio-economic climate in South Africa. This would result in only attempting to manage the disease long after onset of symptoms which may have appeared at a younger age (Al-Mayouf et al. 2021; Weakley et al. 2012). Most participants in this study were female, accounting for 81% of the cohort. These demographics agree with global statistics which identified females to be at higher risk for JIA than males (Al-Mayouf et al. 2021).

All participants, except participant 16, were undergoing treatment and/or busy weaning treatment to achieve or maintain disease remission upon recruitment to the study. The most common line of treatment among participants was the use of corticosteroids, DMARDs and other essential vitamins and supplements. MTX was the most common DMARD across all participant treatments which was used to control disease activity and improve prognosis. Folic acid is used to mitigate gastrointestinal side effects of MTX and was also included in their treatment regimens. In certain cases, biologics such as tocilizumab, an IL-6 inhibitor, would be added where DMARDs alone had failed to manage disease activity and inflammation (Scott and Brice 2015). Most participants had been undergoing treatment for roughly 3-5 years. The goal of treatment is to achieve stable remission in order to wean medication. The risk of relapse however is a major concern when deciding to wean treatment and this highlights the need to identify a reliable marker for detection of subclinical disease activity and risk of relapse (Foell et al. 2004).

5.1.2. Routine Laboratory Measures

FBCs, differentials, CRP and ESR are done routinely when assessing JIA patients at every clinical visit. FBCs and differentials on majority of samples were within normal ranges. The acute phase protein, CRP, was abnormally high in 2.2% of all patient samples while the ESR of 56.8% of patients was higher than normal. The apparent regulation of CRP as opposed to ESR may be because of CRPs sensitivity to the DMARD, MTX, which was a part of all participant treatment regimens (Segal et al. 1989; Murav'ev et al. 2014). The measurement of these acute phase reactants, however, offer no predictive value for the future course of the disease as they reflect a systemic response and correlate poorly with disease activity (Foell and Däbritz 2011).

At visit A and B, most participants were experiencing inactive disease, followed by moderate and low disease activity. The apparent low level of disease activity amongst participants may be due to ongoing treatment with anti-rheumatic drugs which are known to decrease inflammation thereby reducing the number of active joints (Duurland and Wedderburn 2014). Clinical remission is achieved when there are no active joints in conjunction with normal acute phase reactant measurements, and a JADAS score representing inactive disease activity. This was accurate in our findings which identified the active group as having significantly higher numbers of active joints (Consolaro et al. 2016). Cross-sectional evaluation of sampling at visit B identified significantly positive correlations between active joint count and calprotectin among all samples, and within the active disease analysis group. These findings seem to accurately reflect calprotectin being released locally, at the site of joint damage (Kang et al. 2014).

5.2 Monocyte Distribution and Surface Marker Expression

A higher frequency of intermediate monocytes (27%) across all participants at visit A as compared to expected levels (2-5%) was observed in this study. A higher percentage of intermediate monocytes is characteristic amongst patients with autoimmune diseases which implicates them in immune mediated inflammation. In cases of inflammatory disease, TLR-stimulation mediates production of inflammatory cytokines in intermediate monocytes, and this was evident in the study which found the expression of TLR4 to be most frequent on intermediate monocytes when compared to expression levels on the classical and non-classical subsets of all participant samples in this study (Gaur et al. 2016; Marimuthu et al. 2018). However, no correlation was found between calprotectin and TLR4 as described in literature, and this should be explored further in studies which include treatment naïve patients where it likely that levels of calprotectin and TLR4 expression have not yet been dampened.

Intermediate monocyte numbers correlated significantly with the acute phase reactants, ESR and/or CRP, among all participants as well as the matched, disease remission and high ESR analysis

groups, at visit A. This is suggestive of an increase in inflammation and therefore intermediate monocytes, may follow a similar pattern to that of CRP and ESR which are currently the standard markers of inflammation, however poorly specific, when evaluating disease activity in JIA (Foell and Däbritz 2011; Gaur et al. 2016).

Cross sectional analysis of samples from visit B identified significant correlations between TLR4 expression and sCD14 among all samples and within the matched, and high ESR analysis groups. Soluble CD14 has been identified as a non-specific marker of monocyte activation and elevated levels have been associated with inflammatory diseases, including arthritis (Shive et al. 2015). The positive relationship may be attributed to immune activation, whereby TLR4 expression increases in response to circulating cytokines and may likely correlate with the expression of sCD14 upon immune activation in cases of JIA (Vaure and Liu 2014).

The expression of IL-2Rα (sCD25) and TLR4 also showed a positive correlation among multiple groups including the remission group at visit A and all participants, as well as the matched, and high ESR analysis groups visit B. This relationship is likely due to the presence of immune activation: IL-2Rα is released into circulation upon immune activation while TLR4, which is constitutively expressed on monocytes, increases in response to circulating cytokines during immune activation. The feedback loop continues when TLR4 binds circulating DAMPs, such as calprotectin, and induces further production of proinflammatory cytokines (Vaure and Liu 2014; Damoiseaux 2020).

Additionally, expression of the TLR4 surface marker showed a significant correlation with MDC among all the samples and the low ESR analysis group at visit A. Similarly, at visit B, TLR4 correlated significantly with all samples and within the matched, remission, and high ESR analysis groups. MDC is also referred to as the CCL22 chemokine and has been implicated in the process of synovial inflammation in cases of JIA. The expression and release of MDC from immune cells, including monocytes, is highly regulated by circulating inflammatory signals. Both MDC and TLR4 are constitutively expressed by monocytes and are affected by circulating cytokines during activation of immune pathways such as those resulting in inflammation of the joints (Mantovani et al. 2000; de Jager et al. 2007; Vaure and Liu 2014).

Furthermore, TLR4 expression significantly correlated with that of MCP-1 among the low ESR group at visit A and multiple analysis groups at visit B. Another name for MCP-1 is CCL2 and it is involved in the process of inflammation. Monocytes are a major source of CCL2 which plays a role in regulating migration of these immune cells (Deshmane et al. 2009). De Jager et al. identified CCL2 to be elevated in JIA plasma samples when compared to healthy controls, even during apparent remission (de Jager et al. 2007). Given that TLR4 expression is affected by immune activation and subsequent circulation of inflammatory biomarkers, and the role of CCL2 in migration of monocytes

at the damaged joint, the correlation between these variables seems plausible (de Jager et al. 2007; Vaure and Liu 2014).

The expression of CD86 on monocyte surfaces increased significantly from visit A to visit B within the remission analysis group. A decrease in active disease, and subsequently ongoing inflammation and joint damage, can result in migration of circulating monocytes back to the bone marrow. A decrease in circulating monocytes would reduce the amount of detectable CD86 on monocyte surfaces (Shi and Pamer 2014). The findings are therefore contrary to what was expected and may suggest that there is ongoing subclinical monocyte activity.

Cross-sectional analysis identified significantly positive correlations between CD86 and intermediate, as well as non-classical monocytes among the high ESR groups at visit A and visit B. This may be attributed to the expression of this co-stimulatory molecule on monocytes to drive the adaptive immune response by binding T-cell ligands (Pinto et al. 2018). Expression of CD86 and CD64 among the active disease and high ESR groups at visit B also correlated significantly given that both molecules are constitutively expressed on monocytes. Consequently, CD64 expression correlated significantly with numbers of classical and non-classical monocytes among multiple analysis groups (Li et al. 2009). Similarly, CD86 and CD36 showed a positive correlation among multiple analysis groups because CD36, like CD86, is constitutively expressed on monocytes (Huh et al. 1995).

5.3 Inflammatory Biomarkers

Luminex® assay kits prove extremely efficient for analysing multiple variables at one time. Multiple correlations were identified amongst the inflammatory biomarkers in this study apart from CXCL9, CCL3, IL-1β, IL-12 and IFN-γ, which were all excluded from further analysis as sample concentrations of these markers were out of range.

Correlation analysis identified significant relationships between CD163 and IL-2Rα among multiple comparison groups including the matched, active, and remission analysis groups at visit A. As previously discussed, IL-2Rα is released into circulation during immune activation, which occurs in patients experiencing active disease, and expression of CD163 has also been implicated in the initiation of immune activation by driving the inflammatory response (Kowal et al. 2011; Damoiseaux 2020).

Similarly, CD163 correlated significantly with eotaxin, also known as CCL11. This result was identified among all participants as well as the remission, and high ESR groups at visit A. The expression of CCL11 was confirmed to be elevated among JIA patients when compared to a healthy control group in previous studies and its expression is often induced by the presence of TNFα. It is

involved in migration of leukocytes which suggests its role in joint inflammation involving recruitment of leukocytes to the site of joint damage (de Jager et al. 2007; Muller et al. 2015; Skrzypkowska et al. 2022).

Significant relationships were also identified between sCD14 and TNFα, MDC and MIF. The association between TNFα and MIF expression has been confirmed in previous studies regarding JIA cytokine profiles (de Jager et al. 2007). TNFα is a key role player in inflammation which is a characteristic feature across all subtypes of JIA (Zelová and Hošek 2013; Muller et al. 2015). MDC is constitutively expressed by monocytes and is affected by circulating cytokines during apparent inflammation of the joints (Mantovani et al. 2000). Expression of MIF has been implicated in many pro-inflammatory processes including that involved in forms of JIA joint inflammation (Meazza et al. 2002). Given the involvement of activated monocytes in the inflammatory nature of JIA disease, and the correlation between sCD14 and activated monocytes, the relationship between sCD14 and the above-mentioned markers is convincing (Shive et al. 2015; Marimuthu et al. 2018).

Further significant associations were identified between expression of MDC and TNFα, as well as between MDC and MIF. The relationship between these variables were discussed above and all play pathogenic roles in JIA-associated joint inflammation (de Jager et al. 2007).

5.3.1. Calprotectin (S100A8/A9)

Calprotectin was only above the normal range in 20% of all participant samples. Grouped analysis identified that it was above normal in 25% of the active, 10% of the remission, 24% in the high and 15% in the low ESR groups. These statistics are in line with other studies which have confirmed a marked decrease in serum calprotectin levels in patients treated with DMARDs (Nielsen et al. 2017).

A number of participants considered to be in clinical remission, and displaying clinically inactive disease traits, experienced a relapse in disease. They showed evidence of elevated plasma calprotectin reflecting sub-clinical inflammation prior to the relapse. The predictive value of calprotectin has been investigated in relation to risk of relapse, especially in cases of apparent clinical remission (Gerss et al. 2012). Cases with elevated calprotectin will be discussed further in section 5.4 below.

While JIA disease activity is highly dependent on measurements of CRP and ESR, these inflammatory markers are not highly specific and can fluctuate due to many other reasons. Therefore, the last decade has seen the emergence of this novel marker, calprotectin, with higher specificity and correlation to disease activity and inflamed joints (Vaos et al. 2013; Duurland and Wedderburn 2014). Cross sectional evaluation of the matched, remission and high ESR analysis groups at baseline showed a significantly positive relationship between CRP, ESR and calprotectin. Given that disease activity is currently reliant on CRP and ESR we can speculate that calprotectin correlated

with disease activity. The association between calprotectin and disease activity also suggests that levels of calprotectin have the potential to be surrogate markers for monitoring response to treatment and predicting risk of relapse (Duurland and Wedderburn 2014).

Further cross-sectional analysis revealed significant relationships between calprotectin and active joint counts among all samples at visit B, as well as within the matched, and active disease analysis groups. Together with active disease and inflammation among these groups, an increase in involved joints, recruited monocytes, and subsequent release of calprotectin at the affected joints is confirmed in previous studies (Wang et al. 2018; Marimuthu et al. 2018).

The relationship between calprotectin and monocytes, and between calprotectin and IP-10, were trending toward significance. These relationships may be attributed the action of calprotectin binding to monocyte surface TLR4, which induces transcription of IP-10; IP-10 then recruits inflammatory cells, such as neutrophils and monocytes, to the affected joints and drive the inflammatory process (Wu et al. 2021).

Additionally, the relationship between calprotectin and CD86 expression on monocytes was borderline significant in the active disease group only. This study illustrated that the number of active joints were significantly higher in the active disease group compared to the remission group. Active joints are indicative of ongoing inflammation of the joints, and this may explain the relationship between calprotectin and CD86 expression i.e., an increase in circulating monocytes during joint inflammation would increase the amount of detectable CD86 on monocyte surfaces parallel to the increase in calprotectin expression from activated monocytes at the affected joint (Shi and Pamer 2014; Wang et al. 2018; Marimuthu et al. 2018).

5.3.2. The Effect of Treatment on Inflammatory Investigations

MTX was included in all participant treatment regimens and falls into the DMARD category of anti-rheumatic medications. Wallace et al. described 3 stages of disease remission namely: inactive disease, clinical remission on medication, or clinical remission off medication (Wallace et al. 2004). The data collected from patient files indicated that 18% and 27% of recruited participants were within the inactive disease and clinical remission on medication stages, respectively. There were no participants in clinical remission off medication in this study. MTX is the recommended first approach to treatment in most cases of active JIA disease, however, where it proves to be ineffective, additional biologics may be added to the treatment strategy (Duurland and Wedderburn 2014). The emergence of these combination treatment strategies has made disease remission, or prolonged periods of low levels of disease activity, achievable for many JIA patients as evidenced by disease activity of our cohort in which almost 50% had no apparent disease activity.

There were no significant changes in cytokine and chemokine expression in plasma samples among all analysis groups over time which affected our ability to complete Part II of the objectives set out in Chapter 2. Therefore, a limitation of this study was the treatment strategies already in place when participants were recruited. It is suspected that the combination of corticosteroids, DMARDs and biologics used for the treatment of participants had likely ensured that majority of inflammatory processes were already being managed and dampened. MTX has been shown to reduce the production of proinflammatory cytokines significantly even after an initial dose which aligns with the results of this study (Brown et al. 2016).

5.4 Case Studies

The following participants had unique inflammatory profiles, in relation to calprotectin, and circumstances which will be discussed below.

Participant 10

Participant 10 suffered with oligo-JIA. At visit A, clinical evaluation indicated no complaints related to joints and concluded that disease activity was ‘inactive’. At visit B, no complaints were recorded. The patient was in apparent remission on treatment. Evaluation of collected samples however, showed elevated calprotectin, ESR and CRP at both time points. It was confirmed that this patient was non-compliant on medication, which included DMARDs, and suffered a relapse or ‘flare’ of the joints after our sample collection period. The elevated calprotectin and subsequent relapse are in line with literature which describes calprotectin as a marker for detecting sub-clinical inflammation and risk of relapse (Duurland and Wedderburn 2014; Gerss et al. 2012). Furthermore, inflammatory marker concentrations increased parallel to the compliance issue. From baseline to follow up, plasma levels of CRP, ESR, MCP-1, eotaxin, CD163, MDC, IL-2Rα and IP-10 increased which suggests an activated immune response following the pause in treatment (Mantovani et al. 2000; Kowal et al. 2011; Brown et al. 2016; Damoiseaux 2020).

Participant 16

Participant 16 was the only treatment naïve patient recruited to the study. At baseline, she was diagnosed with new onset oligo-JIA. At baseline, clinical assessment identified a swollen, tender ankle combined with limping. Upon recruitment to this study, the patient was not using any anti-rheumatic drugs, that is, treatment naïve. At follow up, no complaints were recorded, and the patient had been on 6 months of combined DMARDs and corticosteroids and had achieved stable remission. This allowed for weaning of these aggressive therapies. Calprotectin levels dropped from normal levels at baseline to extremely low levels at follow up which may be attributed to the action of the combined medication (Nielsen et al. 2017). CRP levels remained normal throughout and no ESR data could be collected for the follow up visit due to clotted samples which were not viable for testing

by the NHLS. Further evaluation of inflammatory biomarkers saw a decrease in plasma concentrations of eotaxin, MDC and MIF from baseline to follow up which may demonstrate the anti-inflammatory action of the combination therapy (Brown et al. 2016).

Participant 20

Participant 20 was diagnosed with RF negative poly-JIA. At baseline she presented with moderate disease activity which included painful and stiff fingers and wrists. Follow up clinical assessment revealed no complaints except for that of the wrists. Plasma samples analysed from both time points indicated high levels of calprotectin, which may be attributed to poor compliance and access to healthcare in this case. Subsequently, this patient suffered a relapse, and this suggests that the high level of calprotectin could be correlated with subclinical inflammation and risk of flare (Duurland and Wedderburn 2014; Gerss et al. 2012). Additional evidence of non-compliance can be seen in the biomarker profile which shows an apparent increase in plasma concentrations of CRP, ESR, eotaxin and MIF from baseline to follow up (de Jager et al. 2007).

Participant 21

Participant 21 suffered with sJIA, arguably the most severe, albeit rare, subtypes of JIA. At baseline, his complaints included that of the wrist, elbow, and hip with moderate disease activity. While his baseline calprotectin was normal, his follow up measurements were extremely high and is in line with the apparent poor compliance on prescribed medication. In addition to calprotectin, levels of CRP and ESR were also elevated which is common in cases of sJIA. Interestingly, this patient's cousin was recently admitted to the clinic where he is being treated for sJIA as well. This suggests a genetic component, which has been investigated in literature for its role in the risk for this autoimmune disease (Hersh and Prahalad 2015). Further inflammatory investigations revealed an increase in plasma sCD14, MCP-1, CD163, IL-2Rα and MIF from baseline to follow up which suggests a relationship between an increase in inflammation and the poor compliance issue (Brown et al. 2016).

Participant 22

Participant 22 has a unique history. After being diagnosed with RF negative poly-JIA, she failed a total of 4 biologics and is finally responding to tocilizumab in combination with DMARDs and corticosteroids. This is the most aggressive case recruited to this study. While her calprotectin was normal at baseline, it was approaching high levels at follow up which concurred with her clinical presentation of a stiff and painful hand, as well as a sore hip and back. In addition to calprotectin, ESR was also high at follow up which can be attributed to moderate disease activity and active joint involvement. This patient also suffered a relapse, and the high levels of calprotectin suggested the presence of sub-clinical inflammation prior to this (Duurland and Wedderburn 2014; Gerss et al. 2012).

5.5 Overall Findings and Limitations

Significant trends could be identified among the calprotectin, inflammatory biomarkers and molecular variables investigated as part of this study through grouped analysis of collected samples. The most prevalent subtypes among participants were oligo- and poly-JIA which was in line with previous studies observing trends in African regions. Similarly, the disease seemed to affect more females than males which agrees with the literature. All participants, except for Participant 16, were already undergoing treatment at the time of recruitment to the study.

The standard markers of inflammation used to monitor disease activity at the Tygerberg clinic were CRP and ESR. The levels of CRP were normal in most patient samples where ESR reflected some inflammatory activity, even during ongoing treatment, and could be used to stratify patients' samples as part of the analysis. These markers, however, offer no predictive value in terms of the future course of disease, specifically in relation to the risk of relapse. This is where calprotectin fills the gap: it is apparently more sensitive in detecting localised inflammation of the joints (though this is to be confirmed in larger studies), it may correlate to risk of relapse and has also been correlated with CRP and ESR in this study making it an ideal marker for monitoring disease activity and risk of relapse in prospective studies.

Cellular analyses identified a clear increase in intermediate monocytes which have been previously linked to inflammatory disorders. Most participants were experiencing inactive disease due to ongoing treatment and consequently showed little to no signs of inflammatory activity which was evident in measurements of the standard markers, CRP and ESR, as well as calprotectin. Where calprotectin was elevated, further investigations suggested a correlation between elevated calprotectin and the risk of relapse as previously mentioned in literature and this should be investigated further in larger longitudinal studies.

Limitations

Given the confounding factor of ongoing treatment with DMARDs and corticosteroids, inflammation and, as a result, serum concentrations of calprotectin did not yield significant results in relation to monitoring disease activity (Brown et al. 2016). It would be useful to recruit treatment naïve patients in future investigations to achieve Part 2 of the objectives for this study which included monitoring response to treatment, and inflammatory biomarker profiles, in relation to disease activity, over time.

Many samples were not collected at visit B due to loss to follow up, miscommunication regarding appointments or time constraints on the study. The small sample size of the cohort, and furthermore JIA subtypes, was a limitation in this study as no PsA, ERA or uJIA cases were included. A more representative sample of the community surrounding TBH could be achieved in future studies by increasing the time in which to recruit participants to the study.

The impact of the ongoing global pandemic was evident in a study such as this. Conducting research at a hospital, which saw the need to visit immunocompromised children, proved difficult given the untimely closure of the paediatric clinic and the inability to collect samples for several months. While the study of scientific topics often throws curveballs, the results of such a study without the effects of a global pandemic and time constraints would be favourable.

Evidently, poor compliance to medications was recorded in a few cases discussed in section 5.4. While this allowed us to observe possible correlations related to calprotectin and sub-clinical disease activity, it highlights the reality of the socio-economic climate in the areas surrounding TBH. Many patients became poorly compliant due to limited access to the necessary resources and care.

Chapter 6 Conclusions and Future Research

Monitoring of JIA disease activity relies heavily on clinical presentation and routine laboratory markers such as CRP and ESR. While these markers are currently the standard practice, they are easily influenced by many other factors. They lack the specificity for localised inflammation, at the synovia, in cases of JIA (Vaos et al. 2013; Duurland and Wedderburn 2014). No relationships were identified between these markers and the number of active joints which may be indicative of a poor correlation between these markers and localised inflammation of the joints.

Calprotectin, which is produced by activated monocytes and macrophages at the site of inflammation, is a pro-inflammatory ligand of TLR4 and has previously been linked to disease activity (Ehrchen et al. 2009). This study found correlations between pro-inflammatory monocytes and TLR4 expression, but this could not be linked to levels of calprotectin. This result may be due to the limitation of ongoing treatment which had dampened inflammation, disease activity, and subsequently expression of calprotectin. Calprotectin has previously been described as a more sensitive marker of disease activity as it detects localised inflammation and is released by infiltrating monocytes. Correlations between calprotectin and the number of active joints in a few analysis groups suggested that it may indeed be more sensitive in detecting localised inflammation at the joints of JIA patients. The correlation between calprotectin, CRP and ESR in a few analysis groups indicates that it may prove useful in the process of JIA diagnosis and monitoring in the clinics where these.

With the ever-expanding pool of research into biomarkers related to childhood diseases, it makes sense that novel predictors of disease course need to be robust, reliable, and cost effective for testing groups attending the clinic every week. Validation of plasma calprotectin in this setting would prove favourable as a predictor of disease outcome.

Pro-inflammatory cytokines have been repeatedly investigated for their role in JIA pathogenesis. The overproduction of these biomarkers leads to the inflammation of joints and subsequent damage. It is no surprise that anti-rheumatic drugs targeting these biomarkers can improve patient outcomes as we have seen in this study (Hahn and Kim 2010). A larger portion of the participants in this study, undergoing treatment and remaining compliant, were in a state of inactive disease, that is, with no active joints due to the apparent anti-inflammatory nature of these drugs and their ability to dampen the production of pro-inflammatory mediators. Furthermore, the extent of these medications' actions could potentially be responsible for the normal plasma concentrations of CRP and calprotectin in most patients with inactive disease.

Disease relapse is a complicated phenomenon and cannot be detected by means of clinical evaluation or testing of routine markers such as CRP and ESR. Calprotectin was amongst few

biomarkers previously identified for its ability to detect even sub-clinical inflammation or residual disease activity in patients believed to be in remission. The measurement of this marker therefore allows paediatric rheumatologists to assess whether treatment can be weaned or not and adds to the scope of individualised therapy (Gerss et al. 2012). The results of this study suggest that calprotectin may detect the risk of relapse, as discussed in section 5.4 relating to case studies. These cases presented with abnormally high concentrations of calprotectin and subsequently suffered a relapse.

There is a delay in diagnosis and disease management in this setting as evidenced by the older average age of patients attending the Tygerberg clinic. This may be attributed to the delay in referral from primary healthcare facilities given the paucity of education, funding and awareness surrounding JIA. These major gaps in South Africa's paediatric rheumatology services need to be addressed to manage the disease more effectively and improve outcomes in these cases (Weakley et al. 2012; Scott and Webb 2013).

Final remarks

It would be useful for future studies in the Western Cape or surrounds to include a larger study cohort to have a more representative sample of the areas surrounding TBH as this study is limited by sample size. Additionally, special attention could be given to including participants newly diagnosed and/or treatment naïve to see the full extent of changes in inflammatory markers and calprotectin before and after therapeutic interventions. Studies such as this may also yield more significant findings if done over a longer period of follow up with additional data points to include in analysis. The results of this study provide valuable insights into the specificity of calprotectin, in predicting the risk for relapse especially in cases of alleged stable remission. The cost of such a test, when done in batches, is reasonable and should be considered as part of the routine work up done at rheumatology clinics in the Western Cape and South Africa to better determine future courses of treatment and to better predict disease outcomes.

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Appendices

Appendix A: Participant assent form

	STELLENBOSCH UNIVERSITY **FACULTY OF HEALTH SCIENCES**	

DO YOU WANT TO TAKE PART IN OUR PROJECT?

NAME OF THE PROJECT:
"Using a protein in your blood to check if our treatment works"

RESEARCHERS IN THE PROJECT:

Ms **Christine** Evert

Dr **Richard** Glashoff

Prof **Monika** Esser

Dr **Deepthi** Raju Abraham

ADDRESS OF THE PROJECT:

Unit of Immunology
Division of Medical Microbiology
Department of Pathology
Faculty of Medicine and Health Sciences
Stellenbosch University
PO Box 241, Cape Town, 8000
South Africa

CELL NUMBER OF THE PROJECT:

082 324 9613

What is this project about?

This project is about doing research about why you are here. Research is something we do to find new knowledge about the way things – and people! – work. We can use research projects to help us find out more about illnesses, for example. Research projects also help us find better ways of treating your illness.

Why are we inviting you take part in this project?

We are inviting you to take part in this project because you have JIA – juvenile idiopathic arthritis, or childhood arthritis.

When you have JIA, your joints (elbows, knuckles, knees, ankles, wrists) are sore, swollen and stiff – in other words, they are inflamed. And, when your joints are inflamed, they make a lot of protein, called calprotectin, and send it into your blood. When your joints are not very inflamed, there is less of this protein in your blood.

How can a protein in your blood help us?

It will help us if we can draw a little bit more of your blood and test it for this protein. We want to see how clearly this protein is linked to your inflamed joints. Maybe this could be a better test for inflamed joints than the tests we already do at the clinic. Maybe treatment could then also be better.

Who is doing the research in the project?

Ms **Christine** Evert is the main person doing the research. She is doing this research to get a master's degree at Stellenbosch University. It is important that she does this research because not a lot of people are studying JIA, or childhood arthritis, in South Africa.

Dr **Richard** Glashoff, Prof **Monika** Esser and Dr **Deepthi** Raju Abraham are her supervisors and are also helping her with her research.

What will happen to you if you take part in the project?

If you take part in the project, the nurse at the clinic will draw a little bit more of your blood than usual. Your extra blood will be used to test how much of the calprotectin protein is in your blood.

Can anything bad happen to you if you take part in the project?

As you know, when blood is drawn, a nurse puts a small needle in a vein in your arm and then fixes a tube to it so blood can run into it. When the nurse puts the needle in your arm, it can feel like a

small pinch. And, when the nurse takes the needle out of your arm, there can be a small bruise. The nurse then puts some cotton wool and a plaster over the bruise.

The nurse will draw 14 ml of blood all in all (as much as would fit in a bottle of nail polish). This is a safe amount of blood for children. Of these 14 ml, 9 ml will be taken for the doctor and 5 ml will be taken for the project.

If you take part in the project and you feel sick or if it becomes too sore, you can tell the nurse or the parent who is with you and you can leave and do not have to take part in the project anymore.

Can anything good happen to you if you take part in the project?

If you take part in the project, you will learn a lot about JIA. We will also give you information leaflets that explain more about it.

You will be able to ask any questions and learn more about the science we are doing. In the future, scientists will be able to use the results of this project to find easier ways to see how well you are responding to your treatment and even see if there is a risk of another inflamed joint developing.

Will anyone know you are taking part in the project?

No one will know you are taking part in the project because we will not use your name. Instead, we will give you a number.

Whom can you talk to about the project?

If you like, you can talk to **Christine** about the project. She is the main person doing the research. You can phone or text her on 082 324 9613 or you can send her a message at 22374507@sun.ac.za.

What if you do not want to take part in the project?

If you do not want to take part in the project, you do not have to. Even if your parents said yes, you can say no. Even if you first said yes and you change your mind, you can then say no. Even if you are already taking part in the project and you want to stop right in the middle of it, you can. And you will not get into any trouble at all!

Do you understand this research project and do you want to take part in it?

YES	NO

Has the researcher answered all your questions?

YES	NO

Do you understand that you can stop taking part in the project at any time?

YES	NO

Do you understand that your results may be used in other projects about JIA but that your name will not be used?

YES	NO

_____ _____

Signature of child Date

Appendix B: Participant consent form

INFORMATION LEAFLET AND CONSENT FORM

TITLE OF RESEARCH PROJECT:	
Calprotectin (S100 A8/A9) as a marker of inflammation and treatment monitoring in cases of juvenile idiopathic arthritis (JIA) in the Western Cape, South Africa	
DETAILS OF PRINCIPAL INVESTIGATOR (PI):	
Title, first name, surname: Ms Christine Evert	**Ethics reference number:** **S19/05/101**
Full postal address: Unit of Immunology Division of Medical Microbiology Department of Pathology Faculty of Medicine and Health Sciences Stellenbosch University PO Box 241, Cape Town, 8000 South Africa	**PI contact number:** 082 324 9613

We would like to invite you to consent to a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are completely satisfied that you clearly understand what this research entails and how you, as a parent/guardian, could be involved. Also, your consent is **entirely voluntary** and you are free to decline to consent. In other words, you may choose to consent or you may choose not to consent. Nothing bad will come of it if you say no: it will not affect your child negatively in any way whatsoever. Refusal to consent will involve no penalty, loss of benefits or reduction in the level of care to which your child is otherwise entitled. You are also free to withdraw from the study at any point, even if you do agree to give your consent initially.

This study has been approved by the **Health Research Ethics Committee at Stellenbosch University**. The study will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, the South African Guidelines for Good Clinical Practice (2006), the Medical Research Council Ethical Guidelines for Research (2002) and the Department of Health Ethics in Health Research: Principles, Processes and Studies (2015).

What is this research study all about?

This is a pilot study at the Unit of Immunology, Stellenbosch University. We are recruiting 20 to 30 patients from the Paediatric Rheumatology Clinic at Tygerberg Hospital who have been diagnosed with any form of JIA.

The principal investigator wants to test the usefulness of calprotectin (a protein in the blood) to measure joint inflammation and response to treatment in cases of childhood arthritis. The reason for selecting such a marker is that it is released directly from the site of joint inflammation and may even detect subclinical inflammation (before a joint is completely inflamed). The protein will be compared to other smaller or similar proteins that are currently used to monitor inflammation of the joints. This will tell us if calprotectin is giving us the same result as methods already being used at the clinic. Not much research has been done in South Africa on JIA and other diseases that cause childhood arthritis. This study is therefore important in contributing to this field of science.

The procedures involved will include a blood draw, together with the routine blood draw of 9mL, at the clinic when the patients arrive for their examination. A 5mL blood tube will be collected for the study and this blood will be divided by the principal investigator for two experiments: the first experiment will test for levels of the calprotectin protein while the other experiment will look at the activated immune cells involved in releasing calprotectin.

No changes to medication or hospital care will take place; the only change will simply be an extra tube of blood taken by the nurse upon the patients' visit to the clinic. In total, 14mL will be drawn when the patients visit the clinic: 9mL for the doctor's tests and 5mL for the research study.

Why are we inviting you to consent for your child to take part?

The study is focused on juvenile patients (16 or younger) who have been diagnosed with JIA. These patients will either be in remission, be newly diagnosed or currently be on treatment. The calprotectin protein levels are expected to be different at the various stages of the disease. Any patients meeting these criteria will be able to join the study. Patients will not benefit from taking part and will not face any consequences for not taking part – it is a completely voluntary study.

What will your responsibilities be?

Your responsibility will include bringing your child to attend the clinical examinations and follow-ups and allowing the nurses to draw some extra blood for the research in this study. The principal investigator involved in this study will be responsible for making sure that the patients understand the research being conducted and that they will be able to drop out of the study if they wish, with no consequences to them or their treatment.

Will you or your child benefit from taking part in this research?

Initially, the study will provide some information on the different types of JIA and what the disease does to the body. This will educate both patients and parents/guardians about JIA and the research being conducted.

There will be no immediate benefit to those participating in the study. In other words, it will not improve treatment and there will be no compensation for participating. However, the results of the study may be used in the future as a novel method of monitoring inflammation.

Are there any risks involved in your child taking part in this research?

The research holds no risk for the patients and will not affect current therapy in any way. An amount of 9mL of blood will be drawn routinely at clinical visits and 5mL of extra blood will be drawn for the study from the same venepuncture (no extra needles will be inserted for the blood draw – there will be only one needle and one puncture in the arm). Some bruising will occur after the needle is removed and the puncture will be covered with some cotton and a plaster.

If you do not consent to your child taking part, what alternatives do you or your child have?

If you do not agree to take part, there will be no consequences. In other words, whether or not you take part in the study will not affect the patient or the therapy in any way.

Who will have access to your child's medical records?

The principal investigator will have access to the patients' medical records. In the case of publication, all the patients' names will be anonymised using a reference system in order to keep the identities of the patients private.

In the case of follow-up studies, the results of this research project will be used for further research. The names of the patients will, however, remain anonymous or private.

Even though it is unlikely, what will happen if your child gets injured somehow because your child took part in this research study?

Stellenbosch University will provide comprehensive no-fault insurance and will pay for any medical costs that came about because patients took part in the research registered with the Health Research Ethics Committee. The patients will not need to prove that the sponsor was at fault.

Will you be paid for your child taking part in this study and are there any costs involved?

There will be no compensation for your child taking part in the study. Patients already attending the clinic will be recruited and no extra time, transport or inconvenience will be necessary.

Do you have all the information that you need?

You may direct any questions that you have to the principal investigator, Ms Christine Evert, at 22374507@sun.ac.za.

Declaration by parent/guardian

By signing below, I, ……..……., agree that the child in my care take part in the research study entitled *Calprotectin (S100 A8/A9) as a marker of inflammation and treatment monitoring in cases of juvenile idiopathic arthritis in the Western Cape, South Africa***.**

I declare that:

- I have read this information and consent form or it was read to me and it is written in a language in which I am fluent and with which I am comfortable.
- I have had a chance to ask questions and I am satisfied that all my questions have been answered.
- I understand that consenting to my child taking part in this study is **voluntary** and that neither I nor my child has been pressurised into consenting or taking part.
- I may choose to remove my child from the study at any time and nothing bad will come of it – my child will not be penalised or prejudiced in any way.
- My child may be asked to leave the study before it has finished if the study doctor or principal investigator feels that it is in my child's best interests or if my child does not follow the study plan that we have agreed on.

Signed at (*place*) .....…..…….. on (*date*) ……..…….. 2020.

……. …….

Signature of parent/guardian **Signature of witness**

Declaration by principal investigator

I *(name),* ……..……, declare that:

- I explained the information in this document in a simple and clear manner to ……..
- I encouraged …….. to ask questions and took enough time to answer them.
- I am satisfied that …….. completely understands all aspects of the research, as discussed above.
- I did/did not use an interpreter. (*If an interpreter was used, the interpreter must sign the declaration below.)*

Signed at (*place*) .....……..…….. on (*date*) ……..…….. 2020.

.....
Signature of investigator

.....
Signature of witness

Declaration by interpreter

I *(name),* ……..……, declare that:

- I have edited the information in this document in a simple and clear manner so that the parents/guardians can understand the information.

Signed at (*place*) .....……..…….. on (*date*) ……..…….. 2020.

.....
Signature of interpreter

.....
Signature of witness

Juvenile idiopathic arthritis (JIA)

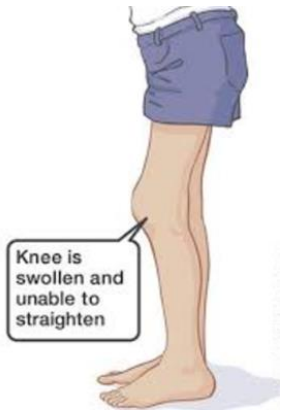
What is JIA?

JIA – or childhood arthritis – is the most common form of arthritis that affects children aged roughly between 2 and 16 years old.

When you have JIA, one or more of your joints – elbows, knuckles, knees, ankles – are inflamed or red and aching. This means they can become sore, swollen and stiff.

There are several types of JIA. These include:

- oligoarticular (most common);
- polyarticular;
- systemic;
- enthesitis-related;
- psoriatic; and
- undifferentiated.



You know which type you have when your doctor has examined your sore joints and looked at your blood tests.

Why do I have JIA?

You have JIA because of a few of reasons: it is in your family (genetics) in combination with past illness and stress on your body. This causes your immune system (the fighters in your body) to become unbalanced and your body to start fighting itself.

How do I get better?

You get better by taking your treatment. This lessens the inflammation and so controls the soreness – which means you can live a better life!

The inflammation in your joints is caused by the presence of small proteins. When we treat JIA, we are actually fighting these small proteins on behalf of your immune system.

The medication we use to treat JIA is called anti-inflammatories, which means “fighting against inflammation”.

You cannot cure JIA – but treatment is important because, if you go on taking your treatment, you will feel much better!

Why have I never heard of JIA before?

You have never heard of JIA before because it is not common enough for people to talk about so people do not know about it.

This is why it is important for YOU to know about it so you can talk about it and teach your family and friends about it.

Many people think only old people get arthritis. No – young people can also get arthritis!

Appendix D: Clinical information for participants at visit A and visit B

Table E- 1: Participant Clinical Background and Routine Laboratory Findings

Patient ID	Age at Recruitment	Sex	Diagnosis	Sample collection date	Clinical Presentation (A & B)	Disease Status (number of active joints)	Disease Flare After Visit	Treatment	Time on Treatment
1 A	14	F	Monoarticular arthritis (oligoarticular JIA) involving the hip	10-Mar-20	R – Painful hip, pending hip replacement	Moderate (1)	n/a	MTX, FA, Sulfasalazine, Brufen (compliant on treatment)	2017-present
1 B									
2 A	15	F	sJIA	22-Sep-20	Hypertension, no complaints	Inactive (0)	N	MTX, FA, VitD, Ca, Enalapril, Brufen (compliant on treatment)	2019-present
2 B*				02-Mar-21	L – Finger trauma unrelated to JIA, no complaints, hypertension	Inactive (0)	N	MTX, FA, Tocilizumab, VitD, Ca, Enalapril	
3 A	19	F	Polyarticular JIA (RF +)	22-Sep-20	History of PTB (bronchiectasis), no complaints	Inactive (0)	N	MTX, FA and HCQ (non-compliant on treatment)	2018-present
3 B*				02-Mar-21	R – wrist affected but no complaints	Low (1)	N	MTX, FA and HCQ (non-compliant on treatment)	
4 A	15	F	sJIA; ANA +; dsDNA +	22-Sep-20	No complaints	Inactive (0)	N	MTX, FA, HCQ (plan to wean MTX)	2015-present
4 B*					No complaints	Inactive (0)	N	MTX, FA, HCQ (compliant on treatment)	
5 A	13	F	Polyarticular JIA (RF +); anti-CCP (+); bilateral uveitis	22-Sep-20	No complaints	Inactive (0)	n/a	MTX, FA, Ca	2019-present
5 B									

6 A	18	F	Polyarticular JIA (RF -)	22-Sep-20	R – painful shoulder, L – painful knee	Moderate (2)	n/a	MTX, Leflunomide, Prednisone, Brufen, Tocilizumab, VitD, Ca	2015-present
6 B									
7 A	8	F	Oligoarticular JIA; ANA (+)	06-Oct-20	No complaints, bilateral uveitis	Inactive (0)	N	MTX, FA	2016-present
7 B*				23-Mar-21	No complaints, bilateral uveitis	Inactive (0)	N	MTX, FA, Zn, VitD, Mtv	
8 A	15	F	sJIA	06-Oct-20	Pin worms seen in stool, in remission	Inactive (0)	n/a		2015-present
8 B									
9 A	15	F	Polyarticular JIA; RF +	06-Oct-20	No complaints	Inactive (0)	N	MTX, FA, HCQ, Enbrel (plan to wean MTX & stretch dose of Enbrel)	2016-present
9 B*				16-Mar-21	No complaints	Inactive (0)	N	MTX, FA, HCQ, Enbrel back to normal (based on bloods), compliant on this treatment	
10 A	15	F	Oligoarticular JIA; ANA (+)	06-Oct-20	JAS, learning difficulties, no complaints, erythematous rash over L molar and under eyes	Inactive (0)	N	MTX, HCQ, Enbrel, VitD, INH	2015-present
10 B*				02-Mar-21	No complaints	Inactive (0)	Y (relapse)	MTX, HCQ, Enbrel, VitD, INH	
11 A	12	F	sJIA	06-Oct-20	Non-compliance and flare-up, destroyed at hip, L – hip pain, Knees & ankles improved on s/c injections	Moderate (1)	n/a	MTX, FA, Tocilizumab, Prednisone, VitD, Ca, Omeprazole (plan to wean Prednisone)	2017-present
11 B									
12 A	15	F	Oligoarticular JIA; enthesitis	06-Oct-20	Abdominal pain, covid positive (3 weeks ago), pain between shoulders, knee pain, knee numbness, lower back pain	Low	N	HCQ, Sulfasalazine	2016-present

12 B*				11-May-21	R – wrist painful on & off, lower back pain	Low (1)	N	MTX, HCQ, VitD, Zn, Mtv (compliant on treatment)	
13 A	13	M	Oligoarticular JIA	07-Oct-20	Simple obesity, mainly L ankle affected	Low (1)	n/a	MTX (additional meds going forward)	2018-present
13 B									
14 A	11	F	Reactive arthritis, new onset JIA (poly RF – JIA); TB (lymphadenitis)	13-Oct-20	R – painful ankle, limping, L – ankle mildly painful	Moderate (2)	n/a	Levofloxin, Linezolid, Pyridoxine, Ethambutol, PenVK	2020-present
14 B									
15 A	6	F	Oligoarticular JIA	13-Oct-20	Suggested JIA, L – Knee	Inactive (1)	N	None currently (old notes)	2019-present
15 B*				09-Mar-21	R – knee pain, traumatic fall, no other joints involved	Inactive (1)	N	MTX, FA, Zn, VitD, Mtv (compliant)	
16 A	2	F	New onset Oligoarticular JIA; ANA +; Bilateral Uveitis	13-Oct-20	L – ankle swollen, tender, limping	Moderate (1)	N	Brufen, Panado	Naïve patient
16 B*				30-Mar-21	R – eye inflamed cells	Inactive (0)	N	MTX, FA, Brufen, Ca, VitD, Zn, Prednisone (wean steroids & add biologics, wean pred)	
17 A	15	M	Polyarticular JIA (RF)	13-Oct-20	Hypothyroidism	Inactive (0)	n/a	MTX, FA, Zn, VitD, Ca	2019-present
17 B									
18 A	10	M	Oligo JIA with uveitis	27-Oct-20	No complaints: uveitis controlled	Inactive (0)	N	MTX, FA; plan to wean MTX	2019-present
18 B*				13-Apr-21	No complaints: uveitis controlled	Inactive (0)	N	MTX, FA, Zn, VitD, Ca, Mtv	
19 A	11	F	Oligoarticular JIA, mild uveitis, ANA (-)	27-Oct-20	FILE NOT FOUND		n/a		2018-present
19 B									
20 A	14	F	Polyarticular JIA (RF -)	27-Oct-20	Fingers painful & stiff in the morning, wrists are tender, poor compliance	Moderate (12)	Y	MTX, FA, Sulfasalazine	2019-present

20 B*				01-Jun-21	No complaints except wrists, apparently compliant		Y	MTX, FA, Sulfasalazine, HCQ, VitD, Zn, Mtv	
21 A	16	M	sJIA	27-Oct-20	Wrist, elbow & hip pain; compliance & supervision a problem	Moderate (8)	Y	MTX, FA, Prednisone, HCQ, VitD, Zn, Mtv	2017-present
21 B*				01-Jun-21	Wrists, and knee limited ROM & tenderness, compliance issues	Moderate (8)	Y	MTX, FA, HCQ, VitD, Zn, Mtv, PenVK	
22 A	18	F	Polyarticular JIA (RF -)	27-Oct-20	R - hand, jaw painful	Low (3)	N	MTX, FA, Tocilizumab, HCQ, Prednisone, Mtv	2016-present
22 B*				13-Apr-21	Fell last week - painful hip and back area; R - hand stiff & painful, MCP limitations.	Moderate (2)	Y	MTX, FA, Tocilizumab, HCQ, Prednisone, VitD, Ca, Mtv	

R – Right, L – Left, MTX: Methotrexate, FA: Folic Acid, VitD: Vitamin D, PenVK: Penicillin, HCQ: Hydroxychloroquine, Mtv: Multivitamin, Zn: Zinc, Ca: Calcium, VitC: Vitamin C, INH: Isoniazid

***Follow up samples collected for measurement of calprotectin and additional biomarkers chosen for this study**

Appendix E: Calprotectin and routine laboratory results for participants at visit A and visit B

Table E- 2: Participant serum calprotectin (S100A8/A9) and routine laboratory findings of baseline (1A-22A) and routine follow up (1B-22B) sampling.

Patient ID	Date of collection	Calprotectin (S100A8/A9) ng/mL	CRP mg/mL	ESR mm/hr	RCC x 10^{12}/L	Hemoglobin g/dL	Platelet Count x 10^9/L	WCC x 10^9/L	Neutrophils x 10^9/L	Lymphocytes x 10^9/L	Monocytes x 10^9/L	Eosinophils x 10^9/L	Basophils x 10^9/L
1 A	10-Mar-20	203.7	<1	9	3.96	12.6	341	6.32	3.41	1.98	0.33	0.42 H	0.04
1 B													
2 A	22-Sep-20	426.3	<1	1	4.35	14.1	251	8.89	5.93	2.27	0.44	0.04	0.04
2 B*	02-Mar-21	1223.8	<1	3	4.44	14.3	298	10.10	5.73	3.31	0.52	0.30	0.08
3 A	22-Sep-20	1369.5	2	30 H	5.00 H	9.4 L	589 H	12.48	9.32 H	2.17	0.60	0.14	0.07
3 B*	02-Mar-21	427.8	1	78 H	4.92 H	9.1 L	639 H	7.75	5.60	1.45	0.45	0.09	0.06
4 A	22-Sep-20	611.2	6	13 H	4.45	13.3	240	6.41	4.13	1.79	0.27	0.10	0.03
4 B*	02-Mar-21	1171.8	7	10	4.03	12.3	245	5.32	2.90	2.04	0.21	0.07	0.02
5 A	22-Sep-20	913.7	1	15 H	3.94	11.9 L	296	7.79	4.71	2.33	0.49	0.12	0.02
5 B													
6 A	22-Sep-20	4858.4 H	<1	8	5.18 H	12.0	290	8.10	5.65	1.43	0.49	0.39	0.06
6 B													
7 A	06-Oct-20	586.5	<1	11 H	4.34	11.8	317	6.93	3.51	2.72	0.24	0.32	0.04
7 B*	23-Mar-21	381.1	<1	17 H	4.34	12.1	302	6.71	3.10	2.46	0.32	0.67	0.07
8 A	06-Oct-20	212.6	1	5	4.53	13.8	350	4.04	1.49 L	1.90	0.39	0.10	0.05
8 B													
9 A	06-Oct-20	421.7	1	25 H	4.85 H	10.6 L	286	4.70	2.32	1.89	0.24	0.15	0.01
9 B*	16-Mar-21	511.8	<1	49 H	4.86 H	10.8 L	276	5.67	3.34	1.91	0.21	0.12	0.02
10 A	06-Oct-20	4079.8 H	34 H	39 H	5.75 H	12.9	418	7.32	4.35	2.01	0.49	0.34	0.04
10 B*	02-Mar-21	2879.3 H	40 H	59 H	5.18 H	12.7	335	8.35	5.00	2.15	0.43	0.63 H	0.04
11 A	06-Oct-20	662.2	<1	NS	4.65	13.6	266	6.25	4.80	1.09	0.19	0.03	0.03
11 B													

12 A	06-Oct-20	646	1	21 H	4.82 H	13.8	350	6.18	4.00	1.70	0.23	0.10	0.03
12 B*	11-May-21	578.7	1	20 H	4.77	13.4	298	8.40	5.86	1.97	0.21	0.22	0.05
13 A	07-Oct-20	456.7	2	25 H	4.68	11.6 L	283	5.35	2.98	1.71	0.41	0.11	0.02
13 B													
14 A	13-Oct-20	359.8	2	15 H	4.37	11.3 L	490 H	8.25	3.37	4.28 H	0.33	0.05	0.02
14 B													
15 A	13-Oct-20	490.4	<1	5	4.72	12.7	314	6.41	2.60	2.88	0.47	0.24	0.04
15 B*	09-Mar-21	994.4	1	12 H	4.62	13.2	329	5.59	2.78	2.04	0.33	0.12	0.08
16 A	13-Oct-20	791.5	1	NS	4.22	11.1	561 H	9.72	3.63	5.13	0.43	0.19	0.06
16 B*	30-Mar-21	101.4 L	5	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
17 A	13-Oct-20	920.7	1	12 H	NS	NS	NS	NS	NS	NS	NS	NS	NS
17 B													
18 A	27-Oct-20	1157	1	15 H	5.52 H	13.9	297	5.67	2.85	2.24	0.33	0.09	0.02
18 B*	13-Apr-21	1069.1	5	28 H	5.40 H	13.3	354	6.51	4.01	1.88	0.35	0.14	0.02
19 A	27-Oct-20	234.2	<1	5	4.47	11.8	309	10.17	6.37 H	2.60	0.45	0.59	0.05
19 B													
20 A	27-Oct-20	1467.1 H	<1	7	4.37	13.1	457 H	8.71	4.45	3.50	0.42	0.09	0.06
20 B*	01-Jun-21	1630.5 H	3	24 H	4.71	12.8	476 H	12.79 H	7.83	3.95	0.54	0.19	0.06
21 A	27-Oct-20	904.2	3	8	5.27	13.0	337	8.33	4.73	3.02	0.32	0.12	0.04
21 B*	01-Jun-21	2937.7 H	28 H	30 H	6.04 H	13.4	359	14.70 H	10.66 H	3.22	0.49	0.09	0.12 H
22 A	27-Oct-20	248.5	<1	8	4.89 H	11.4 L	237	4.24	2.01	1.76	0.20	0.13	0.02
22 B*	13-Apr-21	1379.5	1	36 H	4.83 H	11.3 L	286	6.06	4.06	1.52	0.21	0.12	0.04

RCC: Red Cell Count, WCC: White Cell Count, H: High, L: Low, NS: No Specimen

*Follow up samples collected for measurement of calprotectin and additional biomarkers chosen for this study.
Normal ranges (R&D Systems® Inc. 2015), in children, for calprotectin and routine laboratory findings ((NHLS TrakCare database)) are listed below:

Calprotectin (S100A8/A9)	127	-	1395	ng/mL
CRP		<	10	mg/mL
ESR	0	-	10	mm/hr

Red Cell Count	3.8	-	4.8	x 10^{12}/L
Haemoglobin	12	-	15	g/dL
Platelet Count	186	-	545	x 10^{9}/L
White Cell Count	3.9	-	12.6	x 10^{9}/L
Neutrophils	1.6	-	8.3	x 10^{9}/L
Lymphocytes	1.4	-	4.5	x 10^{9}/L
Monocytes	0.2	-	0.8	x 10^{9}/L
Eosinophils	0	-	0.4	x 10^{9}/L
Basophils	0	-	0.1	x 10^{9}/L

Appendix F: Cross-sectional analysis at visit B

All participants/returning participants.

Figure E-1 illustrates the relationships between the main markers of inflammation, that is, calprotectin (S100 A8/A9), CRP and ESR within this analysis group. There was a significantly positive correlation between calprotectin and CRP (p= 0.0003; r= 0.8440), while CRP and ESR (p= 0.3290; r= 0.3087) and calprotectin and ESR (p= 0.7964; r= 0.0835) did not show any significant correlations. Additional significant relationships were identified among the variables listed in section 4.3; these are summarised in Table E-3 below.

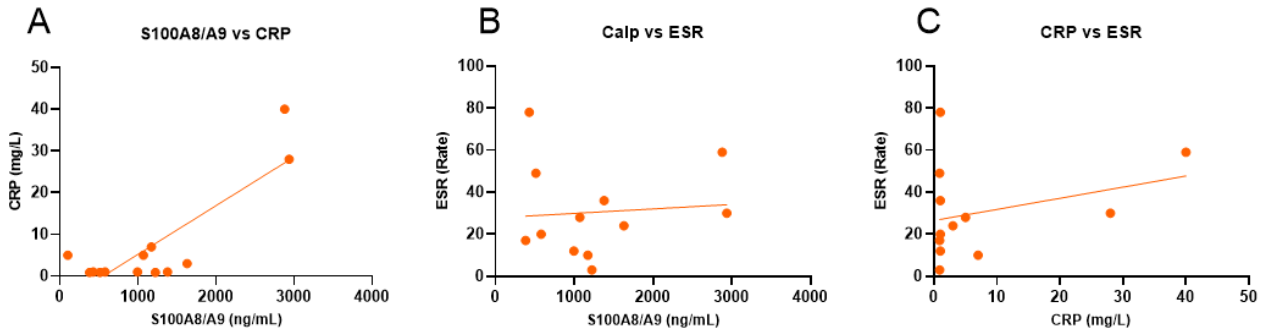


Figure E- 1Scatter plots showing correlations between routine markers of inflammation and S100A8/A9 at routine follow up. A. Calprotectin (S100A8/A9) correlated with CRP (p=0.0003; r=0.8440); B. Calprotectin (S100A8/A9) did not correlate with ESR (p=0.7964; r=0.08350); C. CRP did not correlate with ESR (p=0.3290; r=0.3087). The straight line is a linear regression line to represent the relationship between variables.

Table E- 3: Additional significant correlations between variables among all samples (n=13) at follow up.

Correlation	p-value	r-value
S100A8/A9 vs Active Joint Count	0.0444	0.5879
TLR4 Expression vs sCD14	0.0247	0.6170
TLR4 Expression vs MCP-1	0.0007	0.8165
TLR4 Expression vs MDC	0.0250	0.6160
TLR4 Expression vs IL-2Rα	0.0165	0.6484

Active vs Remission Groups

The following results show significant correlations amongst variables in both the active and disease remission groups at follow up. The relationships between calprotectin and routine inflammatory markers (CRP & ESR) for the active disease group are illustrated in Figure E-2. There were no significant relationships between calprotectin and CRP (p= 0.0683; r= 0.9317), calprotectin and ESR (p= 0.5965; r= -0.4035) or CRP and ESR (p= 0.8268; r= -0.08555). In contrast, significant correlations identified between additional variables of interest are listed in Table E-2 below.

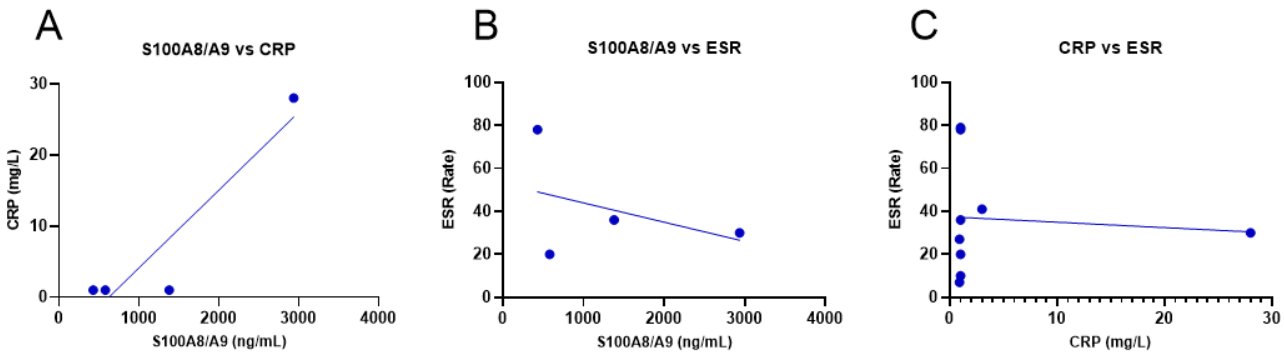


Figure E- 2. Scatter plots showing correlations between routine markers of inflammation and S100A8/A9 at follow up sampling for the active disease group. A. Calprotectin (S100A8/A9) did not correlate with CRP (p=0.0683; r=0.9317); B. Calprotectin (S100A8/A9) did not correlate with ESR (p=0.5965; r=-0.4035); C. CRP did not correlate with ESR (p=0.8268; r=-0.08555).

Table E- 4: Additional significant correlations between variables among the active disease group (n=9) at follow up

Correlation	p-value	r-value
S100A8/A9 vs Active Joint Count	0.0272	0.9728
S100A8/A9 vs Intermediate Monocytes	0.0187	-0.9813
MIF vs ESR	0.0080	-0.9920
Active Joint Count vs CRP	0.0386	0.7800
CD36 Expression vs ESR	0.0300	-0.9700
Intermediate Monocytes vs CRP	0.0346	-0.9654
Classical Monocytes vs Active Joint Count	0.0236	0.9764

The relationships between calprotectin and routine inflammatory markers (CRP & ESR) for the disease remission group are illustrated in Figure E-3. There were significantly positive correlations between calprotectin and CRP (p=0.0080; r=0.8106) and CRP and ESR (p=0.0471; r=0.6727). However, there was no correlation between calprotectin and ESR (p=0.2518; r=0.4597). Significant correlations between additional variables of interest are listed in Table E-3 below.

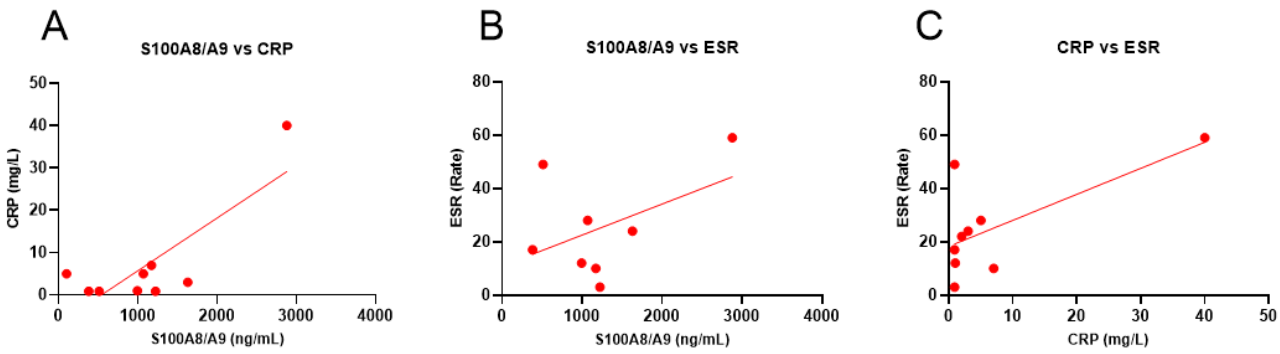


Figure E- 3: Scatter plots showing correlations between routine markers of inflammation and S100A8/A9 at follow up sampling for the disease remission group. A. Calprotectin (S100A8/A9) correlated with CRP (p=0.0080; r=0.8106); B. Calprotectin (S100A8/A9) did not correlate with ESR (p=0.2518; r=0.4597); C. CRP correlated with ESR (p=0.0471; r=0.6727)

Table E- 5: Additional significant correlations between variables among the disease remission group (n=11) at follow up.

Correlation	p-value	r-value
Active Joint Count vs MCP-1	0.0084	0.8438
Active Joint Count vs MIF	0.0022	0.9012
TLR4 Expression vs MCP-1	0.0043	0.8432
TLR4 Expression vs MDC	0.0249	0.7322
Intermediate Monocytes vs IP-10	0.0041	0.8453

Stratified according to ESR measurements

Participant data was stratified according to the rate of ESR to observe any significant trends. The relationships between calprotectin and routine inflammatory markers (CRP & ESR) for the high ESR group are illustrated in Figure E-4. There was a significantly positive correlation between calprotectin and CRP (p= 0.0005; r= 0.8950) and no correlations between CRP and ESR (p= 0.5551; r= 0.1726) or calprotectin and ESR (p= 0.8332; r= 0.0767). Additional significant correlations between variables of interest are listed in Table E-4 below.

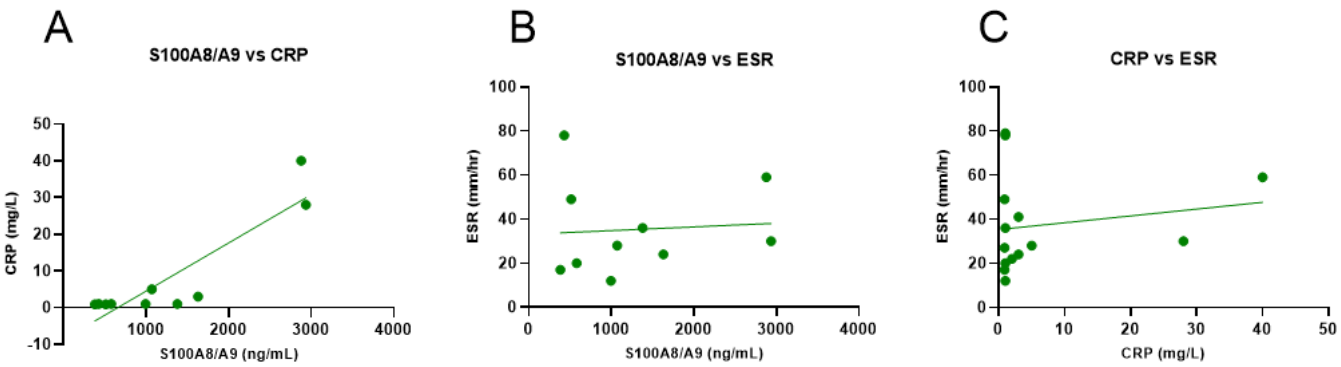


Figure E- 4.Scatter plots showing correlations between routine markers of inflammation and S100A8/A9 at follow up sampling for the stratified group with a high rate ofESR. A. Calprotectin (S100A8/A9) correlated with CRP (p=0.0005; r=0.8950); B. Calprotectin (S100A8/A9) did not correlate with ESR (p=0.8332; r=0.0767); C. CRP did not correlate with ESR (p=0.5551; r=0.1726).

Table E- 6: Additional significant correlations between variables among the stratified group (n=14) with a high rate of ESR at follow up.

Correlation	p-value	r-value
ESR vs Platelet Count	0.0096	0.6643
ESR vs CD36 Expression	0.0164	-0.7306
sCD14 vs TLR4 Expression	0.0328	0.6733
MCP-1 vs TLR4 Expression	0.0025	0.8380
MDC vs TLR4 Expression	0.0109	0.7588
IL-2Rα vs TLR4 Expression	0.0038	0.8184
MIF vs TLR4 Expression	<0.001	0.9629
CD86 Expression vs Intermediate Monocytes	0.0008	-0.8796

ESR data was extremely skewed at follow up, involving only 14 participants with a high and 4 with a low ESR. This data group was excluded from this analysis.

Appendix G: Descriptive statistics for marker expression on monocyte subsets, at both visits

Table E- 7: Descriptive statistics for marker expression on total monocytes and monocyte subsets of all participants whole blood samples (n = 35) analysed using flow cytometry at visit A and B.

	% CD86 Expression Median (IQR)			% CD64 Expression Median (IQR)			% CD36 Expression Median (IQR)			% TLR4 Expression Median (IQR)		
	A	**B**	**p-value**	**A**	**B**	**p-value**	**A**	**B**	**p-value**	**A**	**B**	**p-value**
Total Monocytes	68.4 (2.4 - 85.9)	86.7 (81.9 - 91.1)	0.0942	76.0 (59.7 - 84.7)	83.8 (76.7 - 85.8)	0.1968	86.1 (62.4 - 91.6)	87.9 (80.8 - 92.7)	0.4973	0.8 (0.4 - 2.9)	1.1 (0.7 - 1.5)	0.5759
Classical Monocytes (CD14++ CD16+)	99.4 (2.5-99.9)	99.9 (99.8-100)	0.0739	99.9 (96.8-100)	100 (99.9-100)	0.5473	96.6 (83.4-99.9)	98.8 (95-99.6)	>0.9999	19.9 (6.3-45.4)	30.5 (18-56.1)	>0.9999
Intermediate Monocytes (CD14++ CD16++)	99.8 (17.3-99.9)	99.9 (99.8-100)	0.0655	99.9 (99.8-99.9)	99.8 (99.3-99.9)	>0.9999	99.3 (96.9-99.9)	99.7 (98.6-99.9)	>0.9999	35.5 (21.4-65.4)	45.8 (26.3-65.1)	>0.9999
Non-classical Monocytes (CD14+ CD16++)	40.8 (3.3-70.4)	58.7 (50.2-59.9)	0.8996	80.1 (58.5-89.8)	64.3 (54.6-75.7)	0.8684	87.1 (68.3-94.5)	80.1 (75.3-95.3)	>0.9999	15.2 (10.3-32.4)	18.7 (12.3-37.2)	>0.9999

Appendix H: Descriptive statistics for main variables of interest for active and remission groups at visit A and B

Table E- 8: Descriptive statistics for main variables of interest in the active and remission analysis groups at visit A and visit B.

	Visit A Median (IQR)			**Visit B** Median (IQR)		
	Active	Remission	p-value	Active	Remission	p-value
Total number of participants	10	11		4	9	
Calprotectin (ng/mL)	654.1 (384-876)	611.2 (458-1039)	> 0.999	979.1 (541-1769)	1069.1 (512-1224)	> 0.999
CRP (mg/mL)	1 (0.9-1.8)	1 (1-1.5)	> 0.999	1 (1-7.8)	3 (0.9-5)	> 0.999
ESR (mm/hr)	8.5 (8-16.5)	13 (8-20)	> 0.999	33 (27.5 -46.5)	20.5 (11.5 - 33.3)	> 0.999
Active joints	2 (1-3)	0 (0-0)	0.0012	1.5 (1 – 5)	0 (0 – 0)	0.0023
Total Monocytes (x 10^9/L)	0.3 (0.3-0.4)	0.4 (0.3-0.5)	> 0.999	0.3 (0.2 – 0.5)	0.3 (0.3 - 0.5)	> 0.999
Monocyte Subsets (% total monocytes)						
Classical	41.3 (9-51)	19 (2-58)	0.4335	16.7 (11- 28)	28.9 (13-53)	0.4988
Intermediate	29.7 (26-41)	21.4 (8.2-42)	0.6561	59.4 (50-63)	29.9 (18.5-42)	0.0566
Non-classical	13.5 (8.5- 7.9)	7 (6-15)	0.2109	10.7 (8.9-14.8)	8.9 (6.9-11.1)	0.6708

Appendix I: Descriptive statistics for main variables of interest for high and low ESR stratified groups at visit A and B

Table E- 9: Descriptive statistics for main variables of interest in the high and low ESR stratified analysis groups at visit A and visit B.

	Visit A Median (IQR)			**Visit B** Median (IQR)		
	High	Low	p-value	High	Low	p-value
Total number of participants	11	9		10	2	
Calprotectin (ng/mL)	646 (521.6 - 1038.9)	426.3 (234.2 - 904.2)	> 0.999	1031.75 (528.5 - 1567.8)	1197.8 (1185-1211)	> 0.999
CRP (mg/mL)	1 (1 – 2)	0.9 (0.9 - 0.9)	> 0.999	1 (1 - 4.5)	4 (2.4 - 5.5)	> 0.999
ESR (mm/hr)	15 (14 – 25)	7 (5 – 8)	> 0.999	29 (21 - 45.8)	6.5 (4.8 - 8.3)	> 0.999
Active joints	0 (0 – 0)	1.5 (0.8 - 4.3)	0.1199	1 (0 – 1)	0 (0 – 0)	>0.999
Total Monocytes (x 10^9/L)	0.3 (0.2 - 0.5)	0.4 (0.3 - 0.5)	> 0.999	0.3 (0.2 - 0.5)	0.4 (0.3 - 0.4)	> 0.999
Monocyte Subsets (% total monocytes)						
Classical	3.8 (0.8 - 37.9)	57.1 (41.5-72)	0.0248	21.7 (7.7 – 53)	21.4 (17 - 25.1)	0.4600
Intermediate	29.2 (12.3 - 61.1)	20.3 (5.5- 30.2)	0.1009	37 (19.5 – 61)	34.8 (32 - 37.2)	0.7332
Non-classical	12.8 (6.1 - 16.9)	6.1 (3.6 -11.1)	0.1549	9.6 (7.1 - 11.6)	8.5 (7.6 - 9.4)	0.3109