

The role of neutrophils in autoimmune disease

'Thesis submitted in accordance with the requirements of the University of
Liverpool for the degree of Doctor in Philosophy

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Author's declaration

I declare that this thesis is the result of my own work. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree of qualification. The contribution of others is made explicit where this has taken place.

This research was carried out at the Institute in the Park, Alder Hey Children's NHS Foundation Trust, and the Department of Women and Children's Health, Institute of Translational Medicine, University of Liverpool.

Signature

Printed name

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Abstract

Anna Glaser. The role of neutrophils in autoimmune disease

Background: Juvenile-onset systemic lupus erythematosus (JSLE) is a multisystem autoimmune disease with manifestations being very diverse between individuals. Neutrophils (PMN), the main innate phagocytic cells have been implied to contribute to disease development and show an Interferon-induced gene signatures (IGS) in patients. Contribution of PMN to disease development and patient stratification have mainly been investigated with genomic, transcriptomic, proteomic approaches.

Aims: (1) To explore the ability to stratify patients with autoimmune diseases based on their IGS, including a metabolomics approach using urine and serum. (2) To investigate the phagocytosis-related gene (PRG) signature (PGS) and IGS in PMN of JSLE and healthy paediatric controls. (3) To determine if the PGS is translated onto protein and functional level in PMN of JSLE patients. (4) To determine the influence of factors present in the neutrophil environment including Tumour Necrosis Factor (TNF) α , Interferon (IFN) α , nucleosomes and signal released from apoptosing cells, on the PRG signature.

Methods: Metabolite profiles of serum and urine were obtained from JSLE patients, Juvenile Idiopathic Arthritis (JIA) and paediatric controls using ^1H NMR spectroscopy, bucket tables for serum samples were created with TopSpin® and Chenomx Profiler® whereas urine sample metabolites were annotated and quantified in Chenomx Profiler® for each sample individually. JSLE and paediatric controls were tested for differences in IGS and PGS using real-time PCR, flow cytometry and ELISA. Effects of stimulation with apoptotic supernatant, nucleosomes, IFN α and TNF α on IGS and PGS were also investigated. Phagocytosis was measured using pHrodo coated bioparticles (*S.aureus*, *E.coli*, zymosan) with flow cytometry and confocal microscopy.

Results: JSLE, JIA and paediatric control patients and the JSLE IFN subgroups were distinguishable from each other in a model with partial-least squares discriminant analysis. Urine metabolites built better models than metabolites of serum. Pathway analysis suggested increased inflammation in patients despite low disease activity and differences for metabolites involved in phagocytosis. The PGS genes TLR2 and S100A9 had increased mRNA and protein expression in neutrophils of JSLE patients compared to paediatric control patients. The main difference between JSLE IFN high and IFN low patients was in Fc γ RIIIb, which was only high in IFN high patients. Increased PRG expression was reflected in increased phagocytosis of *E.coli* and zymosan particles. All stimulants tested contributed to the PGS and IGS, in particular the nucleosomes which increased IFN α , IGS expression and triggered S100A8/A9 release.

Conclusions: Factors in the neutrophil cell environment of JSLE patients can contribute to increased expression of the PGS and IGS. Differences between JSLE, JSLE IFN high, JSLE IFN low and paediatric control patients can be measured at the mRNA, protein and metabolite level. Strong differences suggest that IFN high and IFN low patient subgroups may have different aetiopathogenesis and different cytokine profiles.

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Abbreviations

1D CPMG	1D Carr-Purcell-Meiboom-Gill sequence
ACR	American College of Rheumatology
ADP/UDP	Adenosine/uridine diphosphate
ANA	antinuclear antibodies
ANOVA	analysis of variance
anti-dsDNA	anti-double stranded DNA
anti-RNP	anti-ribonucleoprotein
anti-Smith	anti-Smith
AnxA3	Annexin 3
AS	apoptotic supernatant
ATP/UTP	Adenosine/uridine triphosphate
BAFF	B-cell activating factor
Bcl-2	B-cell lymphoma 2 protein
BCR	B-cell receptor
BILAG	British Isles Lupus Assessment Group
BRCA2	Breast Cancer 2
BSA	bovine serum albumin
C3/C4	complement $\frac{3}{4}$
CamK1D	Ca ²⁺ /Calmodulin-dependent kinase 1 delta
CD	cluster of differentiation
cDNA	complementary DNA
CH50	assay to measure the 50% haemolytic complement
CHAQ	Childhood Health Assessment Questionnaire
CMG-2	capillary morphogenesis gene 2

CNV	copy number variants
CR3	complement receptor 3
CRP	C-reactive protein
C _T	cycle threshold
Ctrl	Control
CXCR4	C-X-C chemokine receptor type 4
DAMP	damage associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cells
dsDNA	double-stranded DNA
ECG	electrocardiogram
E.coli	Escherichia coli
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ENA	extractable nuclear antigen
ESR	Erythrocyte sedimentation rate
FADD	Fas-associated protein with death domain
FasL	Fas Ligand
FcγRIIIb	Fcγ receptor IIIb
FCS	foetal calf serum
fMLP	N-Formylmethionyl-leucyl-phenylalanine
FS	forward scatter
g	relative centrifugal force
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor

GWAS	genome-wide association study
h	hour(s)
HBSS	Hanks' Balanced Salt Solution
HCQ	Hydroxychloroquine
HIF	hypoxia-inducible factors
HLA	human leukocyte antigen
HMDB	human metabolome database
HMGB-1	High-mobility group box-1 protein
HRP	horseradish peroxidase
HSV	Herpes Simplex virus type I
IFI44L	Interferon induced protein 44-like
IFI6	IFN α inducible protein 6
IFN	Interferon
IFNAR	Interferon alpha receptor
Ig	immunoglobulin
IG	Interferon-induced gene
IGS	Interferon-induced gene signature
IL	interleukin
IL-1RA	interleukin 1 receptor antagonist
IL2RA	IL-2 receptor subunit alpha
IL2RB	IL-2 receptor subunit beta
ITAM	immunoreceptor tyrosine-based activation motif
JAK1	Janus Kinase-1
JIA	Juvenile idiopathic arthritis
JSLE	juvenile-onset systemic lupus erythematosus

KEGG	Kyoto Encyclopaedia of Genes and Genome
LDG	low density granulocytes
LN	Lupus Nephritis
LPS	lipopolysaccharide
LY6E	Lymphocyte antigen 6 family member E
MALP-2	macrophage-activating lipopeptide 2
MAS	macrophage activation syndrome
MHC	Major histocompatibility complex
MHz	megahertz
min	minute(s)
MMF	mycophenolate
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MS	mass-spectrometry
MyD88	Myeloid differentiation primary response gene 88
Mz	longitudinal magnetisation; z-direction
NaCl	Sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NETosis	NET production
NF- κ B	nuclear factor- κ B
NK-cell	Natural Killer cell
NMR	nuclear magnetic resonance
NOD	nucleotide oligomerization domain proteins
NSAID	non-steroidal anti-inflammatory drugs

OAS1/2	2'-5'oligoadenylate synthetase 1/2
OVN	overnight
PAD4	peptidylarginine deiminase 4
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	principal component
PCA	principal component analysis
PGS	phagocytosis-related gene signature
pDC	plasmacytoid dendritic cells
PI	Propidium Iodide
PLS-DA	partial least squares discriminant analysis
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphomononuclear cells
poly I:C	polyinosinic:polycytidylic acid
ppm	parts per million
PPP	Pentose Phosphate Pathway
PQN	probabilistic quotient normalization
PRG	Phagocytosis related genes
PRKCD	Protein Kinase C Delta
PRR	pattern recognition receptor
PTPN22	protein tyrosine phosphatase, non-receptor type 22
RA	rheumatoid arthritis
RF	Rheumatoid Factor
RNA-seq	RNA sequencing

RNP	ribonucleoproteins
ROS	reactive oxygen species
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
RTase	reverse transcriptase
RT-PCR	Real-time Polymerase Chain Reaction
SAMSN1	SAM domain, SH3 domain and nuclear localization signals 1
SAP30	Sin3A Associated Protein 30
<i>S.aureus</i>	Staphylococcus aureus
SETD7	SET domain containing lysine methyltransferase 7
sJIA	Systemic JIA
SLE	systemic lupus erythematosus
SLEDAI	SLE disease activity index
SLICC	Systemic Lupus International Collaborating Clinics
SNP	Single nucleotide polymorphisms
SRCAP	Snf2-Related CBP Activator Protein
SS	sideward scatter
ssDNA	ssDNA
SYK	spleen tyrosine kinase
TBS	Tris-Buffered saline
TCR	T-cell receptor
T _h	T-helper cells
TLR	Toll-like receptor
TMB	3,3', 5,5' tetramethylbenzidine

TNF α	tumour necrosis factor alpha
TNFR1/2	TNF-receptor 1/2
TGF- β	transforming growth factor beta
TRAIL	TNF-related apoptosis-inducing ligand
T _{reg}	regulatory T-cells
TREX-1	Three-prime repair exonuclease 1
TSA-1	thymic shared antigen 1
TSP	Trimethylsilylpropanoic acid
U/ml	units per ml
UDP	Uridine-diphosphate
UK	United Kingdom
UV	ultraviolet
VIP	Variable Importance in Projection
WB	White British
XRCC4	X-Ray Repair Cross Complementing 4

1 Introduction

1.1 Autoimmune diseases

The role of the immune system is to defend against external pathogens such as bacteria, fungi and viruses. To achieve this there are a number of key defensive and killing mechanisms in place that include processes such as inflammation and antibody production. Environmental factors and genetic predisposition can disturb the homeostasis of the immune system which may lead to autoimmune diseases [1]. In the autoimmune state, the tolerance against self is lost as B-cells develop autoantibodies that target healthy cells. The constant attack and the formation of antibody complexes directed against autoantigens, so called immune complexes, leads to pathology [2]. While in some autoimmune diseases the damage is localized only in one organ, in other autoimmune diseases it involves the whole body, and these are called systemic autoimmune disorders.

1.2 Juvenile idiopathic arthritis (JIA) – An autoinflammatory and autoimmune disease

1.1.1 Clinical manifestations of JIA

JIA is a term used to describe not one disease, but a spectrum of disorders which comprise different subgroups of childhood-onset inflammatory arthritis with unknown aetiology characterised by autoinflammation and/or autoimmunity. Due to these dual characteristics of JIA, it has been used frequently when a comparison is needed to explore an autoimmune disease mechanism characterized by inflammation [3], [4]. Diagnosis of JIA is based on a set of inclusion and exclusion criteria, based predominantly on clinical features in which symptoms of arthritis need to be present for longer than six weeks and diagnosis needs to be before the 16th birthday [5].

The classification of JIA is divided into seven subtypes. This comprises: oligoarthritis (sub-divided then into persistent and extended oligoarthritis), polyarthritis rheumatoid factor negative, polyarthritis rheumatoid factor positive, psoriatic-associated, enthesitis-related, undifferentiated and systemic

arthritis [5]. The different subtypes are listed in **Table 1.1** together with their inclusion and exclusion criteria.

Type of arthritis	Inclusion criteria	Exclusion criteria
Oligoarthritis - Persistent - Extended	Arthritis in 1-4 joints (during the first six months from disease onset) Affected joints remain less than four thereafter ≥ 4 joints after the first six months	1), 2), 3), 4), 5)
Polyarthritis RF-negative	≥ 5 joints during first six months RF test negative	1), 2), 3), 4), 5)
Polyarthritis RF-positive	≥ 5 joints during first six months RF test positive ≥ twice at least three months apart during first six months of disease	1), 2), 3), 5)
Systemic arthritis	Arthritis in ≥ 1 joints Fever > 2 weeks duration + One of the following: A) Evanescent rash B) Lymph node enlargement C) Hepatomegaly and/or splenomegaly D) Serositis	1), 2), 3), 4)
Psoriatic arthritis	Arthritis and psoriasis Or Arthritis + two of the following: A) Dactylitis B) Nail pitting or onycholysis C) Psoriasis in first-degree relative	2), 3), 4), 5)
Enthesitis-related arthritis	Arthritis and enthesitis Or Arthritis or enthesitis + ≥ two of the following: A) Past of sacroiliac joint tenderness and/or inflammatory lumbosacral B) HLA-B27 positive C) If male, arthritis ≥ 6 years of age D) Acute (symptomatic) anterior uveitis E) Ankylosing spondylitis, enthesitis related arthritis, sacroiliitis with inflammatory bowel disease, Reiter's syndrome or acute anterior uveitis (or family history in first degree relative)	1), 4), 5)
Undifferentiated arthritis	If no other category or several can be assigned	

Table 1.1: Overview of subtypes of juvenile arthritis. The seven subtypes of JIA are summarised in this table including their diagnosis criteria. Exclusion criteria are abbreviated in the table as numbers 1-5: 1) Diagnosis of psoriasis (or family history in first degree relative) 2) Arthritis if HLA-B27 positive male (> 6 years) 3) Ankylosing spondylitis, enthesitis related arthritis, sacroiliitis with inflammatory bowel disease, Reiter's syndrome or acute anterior uveitis (or family history in first degree relative) 4) IgM rheumatoid factor (RF) ≥ twice at least three months apart 5) Presence of systemic JIA (sJIA) in the patient.

Heterogeneity of symptoms and disease development may hinder clear classification of the disease subtype, as well as prediction of active or inactive disease state. Being able to accurately predict disease state and treatment response remains a significant problem and challenge, despite extensive prediction models having been developed including a range of clinical parameters, imaging modalities and biological data including genetic, molecular, proteomic, as well as microbiota samples. The current clinical classification criteria of JIA into specific subtypes allows development of moderately effective prediction models [6], but there is still a considerable paucity of understanding in this area. Despite these differences, most patients are managed with the same or similar standard treatments and treatment protocols, such as intra-articular corticosteroid injections with or without non-steroidal anti-inflammatory drugs (NSAID), and/or methotrexate with or without the addition of systemic corticosteroids or the newer biological agents.

Within the different JIA subtypes, systemic-onset JIA (sJIA) is considered to be one of the most severe types. Even with new biologic therapies the burden on patients and their caregivers is extremely high. sJIA patients have reduced physical function, their social lives are affected, and they often need at a young age an assistive mobility device which may be only a walking stick but can be a wheelchair [7].

A relatively common and very severe complication associated with sJIA is macrophage activation syndrome (MAS). Symptoms are severe and include fever, hepatosplenomegaly, lymphadenopathy, cytopenia, coagulopathy and inflammation of the central nervous system. MAS is difficult to diagnose and can be detected more easily in bone marrow aspirates which can be obtained only invasively [6]. Nevertheless, diagnosis is essential as a complication with MAS can need critical care intervention in an intensive care unit in case of severe deterioration. Biologics are a new generation of treatment which are derived from humans, animals or microorganisms and an example are antibodies targeting specific cytokines. This treatment has benefitted JIA patients, but despite intensive care intervention and disease management with biologic drugs about 8% of sJIA patients die of MAS [8].

1.1.2 Pathogenesis of JIA

Already the name idiopathic arthritis implies that there is no known cause for the onset of the disease. This shows how poorly understood this disease is and that there is not much known about its triggers. A starting point to explore diseases is to investigate the genomic background.

One common risk allele for JIA is coding for the human leukocyte antigen (HLA) which represents the human form of the major histocompatibility complex (MHC). This protein is important for the presentation of peptides to T-cells and activation of the immune system. It was suggested that the different subtypes of JIA have specific variants of HLA markers. In one study, HLA-DRB1 was linked to oligoarthritis and RF-negative and -positive polyarthritis, while *HLA-DRB1*11* was connected to sJIA and *HLA-B*27* for enthesitis-related arthritis [9].

Further, non-HLA genes have been linked to oligoarthritis and RF-negative polyarthritis. These include genes encoding for protein tyrosine phosphatase, non-receptor type 22 (PTPN22), IL-2 receptor subunit alpha (IL2RA) or beta (IL2RB), all important for T-cell stimulation and therefore indicating importance of these cells [10]. Other studies additionally found capillary morphogenesis gene 2 (CMG-2) and C-X-C chemokine receptor type 4 (CXCR4) to be linked to JIA [11]. CMG-2 regulates extracellular matrix collagen type IV [12] which may explain the decreased synovial lining with type IV collagen found in rheumatoid arthritis (RA) patients [13] and may apply for the juvenile form of the disease. CXCR4 is expressed on cells of the innate and adaptive immune system and is involved in cell growth and migration [14] and may explain abnormal migration into joints. A genome-wide association study (GWAS) in 2017 revealed association of Janus Kinase-1 (JAK1) with oligoarticular JIA and RF-negative polyarticular JIA [15]. This is of special interest as there are JAK inhibitors available, for example Tofacitinib, which is consequently used for treatment in JIA [16]. If the different subtypes of JIA are studied as one group, it may decrease the chances of finding disease relevant genes. For sJIA it has been suggested that there is a distinctly different genetic background compared to other JIA subtypes such as oligoarthritis and polyarthritis [17].

A difference between the type of immune cells involved in sJIA and the other JIA subtypes has also been found. The innate immune system is dominant in sJIA pathogenesis with neutrophils and monocytes infiltrating the tissues and causing inflammation [18]. Also in other JIA subtypes the innate immune system plays a role as low-density granulocytes (LDG) have been found in higher numbers than in healthy controls [19]. JIA pathogenesis is indeed considered to be driven by the adaptive immune system, and primarily by proinflammatory T-helper cells (T_h) called T_h -17 due to their ability to produce Interleukin (IL)-17. The percentage of IL-17 producing cluster of differentiation (CD) 4 positive cells as well as CD3 positive cells is higher both in active and inactive JIA [20]. These cells produce IL-17A and Interferon (IFN) γ and the percentage of T_h -17 cells in the synovium of JIA patients positively correlated with disease activity measured as C-reactive protein (CRP) [21]. This further supports the importance of this cell type. IL-17 is a cytokine triggering further inflammation by stimulation of tumour necrosis factor alpha ($TNF\alpha$) and IL-1 β [22]. $TNF\alpha$ plays an important role in the disease and its inhibition is used as for a treatment [23]. While inflammation is stimulated by T_h -17 cells, T-cells which dampen immune responses called regulatory T-cells (T_{reg}) are impaired [24].

1.3 Juvenile-onset Systemic Lupus Erythematosus (JSLE)- A systemic autoimmune disease

1.3.1 Clinical manifestation of JSLE

Perhaps the most archetypal systemic autoimmune disorder is systemic lupus erythematosus (SLE) which is a disease with a wide range of symptoms. If a patient is diagnosed at an age younger than 17 the disease is considered childhood-onset of Juvenile-onset systemic lupus erythematosus (JSLE). Different clinical manifestations of JSLE are observed. These can vary widely between patients and can range from mild through to life-threatening severe complications. Skin manifestations are common, but all organ systems can be affected. JSLE patients particularly often have major haematological, renal or neurological involvement [25]. Consequently, it is named a systemic

autoimmune disease and the most common symptoms are summarised in **Figure 1.1**.

Clinical parameters are characterised by antibodies against nuclear-autoantigens including single and double stranded DNA (ssDNA and dsDNA), nucleosomes or nuclear proteins such as ribonucleoproteins (RNP) [26].

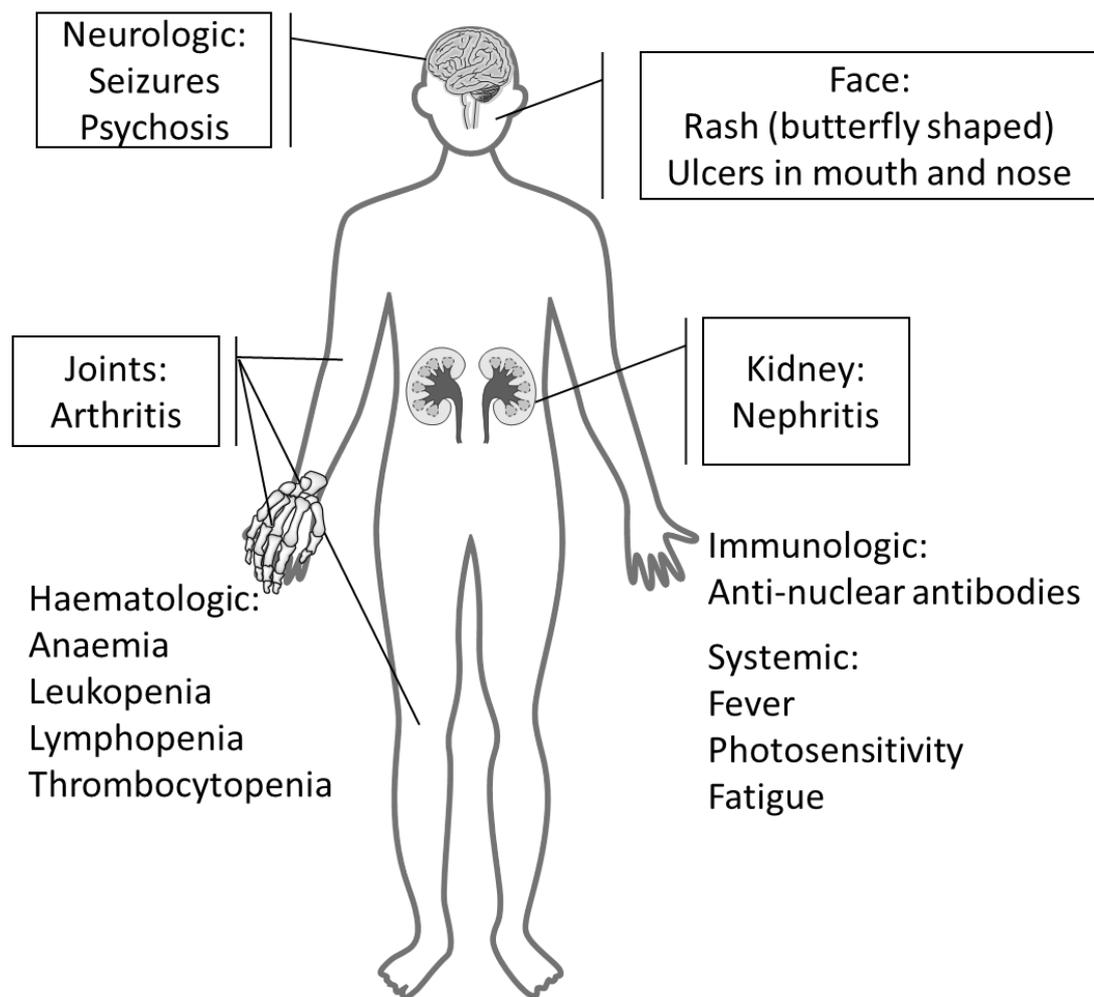


Figure 1.1: Symptoms common for JSLE.

1.3.2 Diagnosis of JSLE and assessment of disease activity

For diagnosis of SLE the American College of Rheumatology (ACR) revised criteria for SLE classification are frequently used. If at least four of the criteria are met before the age of 17, a patient can be diagnosed with JSLE. Symptoms can be present simultaneously or over many years. There are eleven criteria in total, namely: malar rash, discoid lupus, photosensitivity, oral or nasal ulceration, non-erosive arthritis, serositis, nephritis, neurological symptoms (seizures or psychosis), haematological symptoms (leukopenia or lymphopenia on \geq two occasions or thrombocytopenia), immunological symptoms (anti-DNA,

-Sm, -phospholipid antibodies, positive lupus anticoagulant, false positive for syphilis for > six months), abnormal titres of anti-nuclear antibodies [27].

A more recent approach of diagnosis includes these symptoms as the basis as described in the 2012 proposed Systemic Lupus International Collaborating Clinics (SLICC) criteria [28]. The SLICC classification criteria include both clinical and immunological criteria (shown in **Table 1.2**) and in order to confirm a patient to have JSLE, at least four of them have to be met, with at least one immunological and one clinical criterion. Alternatively, it is sufficient for diagnosis if a patient is confirmed with lupus nephritis (LN) by biopsy while antinuclear antibodies (ANA) or anti-double stranded (ds)DNA antibodies are present [28]. SLICC criteria have been shown to be more accurate and sensitive compared to the ACR criteria [29]. Currently, there are new ACR-endorsed Criteria for Rheumatic Diseases, but they have not yet been assessed for JSLE compared to the older classification systems.

Clinical Criteria	
Acute cutaneous lupus	Lupus malar rash Bullous lupus Toxic epidermal necrolysis variant of SLE Maculopapular lupus rash Photosensitive lupus rash Subacute cutaneous lupus
Chronic cutaneous lupus	Classical discoid rash Hypertrophic (verrucous) lupus Lupus panniculitis (profundus) Mucosal lupus Lupus erythematosus tumidus Chillblains lupus Discoid lupus/lichen planus overlap
Oral ulcers	Palate, buccal, tongue or nasal in the absence of other causes (e.g. infection with herpes)
Nonscarring alopecia	In absence of other causes e.g. iron deficiency
Synovitis	Involving \geq two joints, (swelling or effusion) OR tenderness in \geq 2 joints and \geq 30 minutes morning stiffness
Serositis	Typical pleurisy > one day OR pleural effusion OR rub Typical pericardial pain > one day OR pericardial effusion OR rub or pericarditis by ECG
Renal	Urine protein 500mg of protein/24 h
Neurologic	Seizures Psychosis Mononeuritis multiplex Myelitis Peripheral or cranial neuropathy Acute confusional state
Haemolytic anaemia	
Leukopenia	< 4000/mm ³ at least once
Lymphopenia	< 1000/mm ³ at least once
Thrombocytopenia	< 100,000/mm ³ at least once
Immunological Criteria	
ANA	Above laboratory reference range
Anti-dsDNA	Above laboratory reference range (ELISA twice above laboratory reference range)
Anti-Sm	
Antiphospholipid antibody	Lupus anticoagulant OR false-positive RPR OR medium/high titre anticardiolipin OR anti- β 2 glycoprotein I
Low complement	Low C3 OR C4 or CH50
Direct Coombs test	

Table 1.2: SLICC criteria for diagnosis of SLE

The disease is characterised by periods of quiescence as well as periods of active disease which are called flares. During flares, patients may suffer from increased pain, fatigue or rashes, also increased sensitivity to ultraviolet (UV) light, headaches, fever or other organ manifestations. Multiple factors including stress, UV light exposure and intercurrent infections have been suggested to cause such flares [30].

The disease activity, which can affect any organ system, may be assessed using specific lupus disease activity scores. These include the British Isles Lupus Assessment Group (BILAG)-2004 index or the SLE disease activity index (SLEDAI)-2K disease activity score.

The BILAG-2004 index evaluates the nine following organ systems: constitutional, mucocutaneous, central nervous system, musculoskeletal, cardiovascular/respiratory, abdominal, renal, ophthalmic and haematological. Each system is graded separately from A (active) to D (inactive now) or E (never been active). A total score (maximum score 108) is then calculated replacing the letters with numerical values (A=12, B=8, C=1, D=0, E=0) [31]. It is a comprehensive multi-organ assessment and linked to the clinician's intention to treat, which shows *inter-rater* reliability and thus is a reliable tool for assessing disease activity [32]. The SLEDAI-2K is a sole numerical system and each symptom is weighed differently with a range of 0-55 as a total score. For example, while presence of fever equates with 1 point, low complement equates with 2 points and a seizure would receive a score of 8 points. If a symptom is present at the time of visit or was present the ten preceding days, its value is accounted for and the sum of all symptoms represents the SLEDAI-2K of the patient. The index focuses on disease activity and excludes previous disease or therapy caused damage [33].

1.3.3 Epidemiology of SLE and JSLE

A recent study reported a reliable incidence of 4.91 per 100,000 people per year (from 1999-2012) and summarized broad disease categorisation data for SLE in the UK. SLE is considered a rare disease and it is 5.8 times higher in females

compared to males when looking across all age groups. The highest incidence appears to be in individuals between 40-49 for women and 60-69 for men. Furthermore, Black Caribbean ethnicity appears to have a higher incidence rate than white people [34]. Children were also included in this study and while only 0.19 per 100,000 children were newly diagnosed at the ages 0-9, from the ages 10-19 1.92 new cases per 100,000 children were found per year. [34]. Each country has different ways of registering patients and patients may be diagnosed incorrectly, both leading to incorrect and potentially low numbers. Incidence of JSLE has been reported to range from 0.47 for example in Japan to 0.9 per 100,000 in Wisconsin in the United States [35].

1.3.4 Pathogenesis of SLE

Reasons for SLE development are still not fully understood and environmental factors, genetic predisposition, hormonal influence as well as medication have been considered as causative factors. Sunlight is an example of a possible environmental trigger, as a higher risk to develop SLE was associated with outdoor working and being exposed to daylight for more than 20 h/week over 2 months/year. Patients were also found to react more often with a blistering sunburn or a rash [36].

Hormones have been considered relevant in the development of SLE due to the increased risk of the disease in females. Furthermore, this hypothesis is supported as oestrogen-receptor stimulation has been shown to shorten survival of lupus-prone mice [37]. In addition, some drugs have been found to induce SLE. Patients have, for example, developed SLE after procainamide treatment which is used as an anti-arrhythmic drug. The effects were not long-lasting and disease ameliorated after the drug was discontinued [38].

Almost 50 years ago, a genetic predisposition to SLE was found to be linked to the HLA Class I B8 [39]. Since then, many other risk alleles have been found. Genes with importance for the complement system such as C4A and C4B are present in lower copy numbers in SLE patients compared to in healthy controls [40], [41]. Furthermore, genes responsible for DNA processing, such as Three-

prime repair exonuclease 1 (TREX1), an exonuclease which processes DNA [42], have been found to be changed in SLE. Deficiency of this protein could increase presence of single-stranded DNA. Monogenic diseases have given insight into genes important for JSLE such as DNASE1L3, responsible for clearance of chromatin [43] and Protein Kinase C Delta (PRKCD) which regulates lymphoproliferation and spontaneous cell death [44].

SLE is a heterogenous disease according to symptomology, but also in the variety of immune cells involved in its pathogenesis. B-cells lose their tolerance, become autoreactive and produce autoantibodies. B-cell activating factor (BAFF) is overexpressed in SLE patients which stimulates the development of autoreactivity [45]. Therapeutically, anti-BAFF antibodies (Belimumab) are now used for treatment of SLE, but the disease still cannot be completely treated with this agent. In a continuation study of a Phase III Belimumab SLE cohort study 10% of patients had to be withdrawn from a Belimumab treatment and 20.6% still experienced a severe flare despite receiving Belimumab [46]. Similarly, anti-CD20 antibodies called Rituximab, target B-cells and deplete them. SLE disease improvement was again not sufficient to consider B-cells the main actors in this disease [47].

For SLE both the adaptive and the innate immune system are considered to play a role in disease onset and progression. Most published studies limit their research to a specific immune cell type, rather than on specific immune-mediated processes including cell death (see Section 1.4.4.4) and especially the process of neutrophil-specific NETosis (see Section 1.4.4.4.3), the phagocytosis of pathogens and how the immune system deals with apoptotic debris (see Section 1.4.4.5).

One of the challenges in studying the immunopathogenesis of JSLE, its causes, risk factors and disease progression is due to the heterogeneity and complexity of the disease and the different factors potentially influencing the outcome as summarised.

1.4 Strategies to stratify SLE patients

For both investigation of the disease and in seeking to target and successfully develop novel drugs for treating SLE, it has been necessary to stratify patients into different SLE clinical subtypes. Strategies include looking only at specific organ involvement, for example LN, or categorising patients depending on disease activity score or ethnicity [48]. Other types of stratifications include separation into adult and juvenile-onset disease, or a more recent approach is division based on the patient's IFN score (as will be discussed further below – see Section 1.4.2).

1.4.1 Adult- and juvenile-onset SLE

Depending on the age when the disease is diagnosed it is divided into two subgroups, which are adult-onset SLE and JSLE. The latter includes all patients who are diagnosed before 18 years of age [49]. Affected children typically show fever, renal involvement, haemolytic anaemia, and neuropsychiatric manifestations (such as encephalopathy) more often than in the adult-onset disease [50]. There are currently limited published data on the disease characteristics but in particular disease pathogenesis in childhood compared to the adult-derived literature. This needs to be addressed not only because the development of JSLE is generally more severe than for adult-onset SLE, but also, these patients make up 15-20% of all lupus patients. JSLE patients do not necessarily have the same cause of disease or disease progression. JSLE is associated with a higher risk of proteinuria, anti-dsDNA, arthritis and leukopenia and early development has a stronger association with risk alleles than adult-onset [51]. Leukopenia and arthritis are more present with advancing age, although this may simply reflect an average older population where the prevalence of arthritis would be higher (related to ageing) than in children [52]. However, levels of anti-dsDNA, anti-Smith (anti-Sm) and anti-ribonucleoprotein (anti-RNP) autoantibodies, along with prevalence of nephritis, which is a very severe manifestation, are higher in JSLE compared to adult-onset SLE patients [52]. These and other studies describe increased

disease severity in the early-onset group. While being potentially a very damaging disease, fewer comorbidities in the younger age group makes juvenile-onset patients a good group to study the mechanism of SLE. Understanding the disease in children will provide important insights into disease pathogenesis and identify potentially more appropriate and safer treatments. This would help to improve lives of these patients as they suffer physiologically and psychologically lifelong from both the disease and the treatment [53]. Differences in disease development and progression make it essential that patients are distinguished between juvenile- and adult-onset groups.

1.4.2 IFN induced gene signature stratification

Recent interest has focussed on the potential for disease stratification based on the patient's expression of IFN-induced genes (IG). In 2003, it was reported that peripheral blood mononuclear cells (PBMC) from 50% of the studied SLE patients were characterised to have an IFN-induced gene signature (IGS), which could not be confirmed to relate to IFN α , IFN β or IFN γ levels in patients' serum and plasma [54]. Patients with a high IFN score based on the IGS were classified as IFN high and patients with an IFN score similar to controls were considered IFN low. Furthermore, this study found that the IGS was indicative for a more severe manifestation of the disease as more IFN high patients were suffering from more renal and/or CNS involvement and hematologic involvement [54]. At the same time another paper was published about the IGS in PBMCs of JSLE patients [55]. The data showed that for most patients studied, their PBMCs displayed an IFN high signature and that this signature had similarities to IFN α stimulated PBMCs of healthy adults. Interestingly, data arising from this study noted in addition an augmented granulopoiesis signature (increased expression of genes promoting granulocyte production and function) linked to IFN high patients, and showed an association between the presence of LDG and granulopoiesis signature. This suggested a link between neutrophils, also called polymorphonuclear cells (PMN), and the IFN high signature. The analysis included a JIA patient group, but an IGS could not be detected for these

individuals [55]. Later published data did identify an IGS in JIA patients, but only in a 30.8% of patients studied [56]. In this study of JIA patients, the IFN high signature was linked to neutrophils from whole blood which would include LDG [56]. Other studies have identified an IGS in patients with juvenile dermatomyositis, undefined interferonopathies and some autoinflammatory diseases [57]. Despite the suggested link to increased disease severity, there is still a significant gap in understanding the effects and causes of the IFN high and low signature in SLE. This is particularly interesting, as most studies to date have investigated IGSs rather in relation to intercurrent viral infections.

Importantly, JSLE patients with differing IGS subgroups (e.g. high or low expression) may have distinct pathoetiological differences and therefore may potentially respond differently to different treatments. Understanding the role of the IGS in the aetiopathogenesis of JSLE and its potential impact on treatment needs further investigation.

Genes that were linked to IFN in the past or even used as part of an IGS were used as part of the IGS for this study. They were further selected based on high mRNA expression in our JSLE cohort (unpublished data). The genes included were: Interferon induced protein 44-like (IFI44L), 2'-5'oligoadenylate synthetase 2 (OAS2), IFN α inducible protein 6 (IFI6) and Lymphocyte antigen 6 family member E (LY6E).

1.4.2.1 Interferon induced protein 44-like gene

One of the key IGS genes is IFI44L. This gene has previously been used as part of an IFN score, a system to evaluate if a patient is IFN high or IFN low based on the average of several genes [57]. It has also been studied as a biomarker for renal damage in SLE as hypomethylation of the promoter region of IFI44L which increases its mRNA expression, has been found to be more common in SLE patients with renal involvement [58]. This coincides with the higher renal involvement found in IFN high patients compared to IFN low patients [54]. The function of IFI44L is still unknown, but its expression has been linked to response to viruses. Human monocyte-derived macrophages upregulate this

gene after infection with recombinant adenovirus expressing HIV-1 protein Vpr, although no specific function has been identified to date [59].

1.4.2.2 2'-5'oligoadenylate synthetase 2 gene

In a similar manner to IFI44L, 2'-5'oligoadenylate synthetase 2 (OAS2) gene expression has been shown to be increased with infection, although this increase was less marked [59]. In contrast to IFI44L, the protein associated with OAS2 has been studied to some extent. It has been found, that OAS2 produced after IFN stimulation remains inactive until it encounters dsRNA [60]. The enzyme activates Ribonuclease L (RNase L) which is capable of degrading viral RNA but also mRNA and rRNA [61]. The OAS2 gene is considered an indicator for the IFN-pathway activation [62] and has previously been used as a predictor for response to IFN α treatment in SLE patients, indicating its potential importance as part of the IGS [63].

1.4.2.3 IFN α inducible protein 6 gene

The IFI6 gene, also called G1P3, has been proposed to be included as part of an IFN scoring system, which can distinguish SLE patients with high expression from rheumatoid arthritis patients with low expression [64]. In contrast, another study has demonstrated increased mRNA levels of IFI6 in early and chronic RA. Furthermore, autoantibody levels against carbamylated protein were correlated to gene expression [65]. IFI6 expression has been correlated to a pro-apoptotic effect in myeloma cells when treated with IFN α -2b. It has therefore been proposed that it can stabilize the mitochondrial potential which would suggest a pro-apoptotic effect also for other cells [66].

1.4.2.4 Lymphocyte antigen 6 family member E gene

Another IFN-induced gene is known as Retinoic acid induced gene E (RIG-E), thymic shared antigen 1 (TSA-1) or also Lymphocyte antigen 6 family member E

(LY6E). TSA-1 has been explored in mice as an important protein for T-cells and their development [67].

LY6E has been used as part of an IFN score together with 2'-5'-Oligoadenylate Synthetase 1 (OAS1), 2'-5'-Oligoadenylate Synthetase Like (OASL), MX Dynamin Like GTPase 1 (MX1), and Interferon-stimulated gene (ISG15) and showed higher prevalence in peripheral blood of SLE patients compared to healthy controls and RA patients [68]. Patients who had active disease, a severe disease activity or who had anti-dsDNA antibodies were found to present with a higher IFN score. While the IFN score was higher in patients with kidney involvement, specifically LY6E was shown to increase with higher proteinuria and kidney manifestation, both inactive and even more so for active nephritis [68].

1.4.3 “-Omics” approaches to investigating JSLE immunopathogenesis

The term “omics” is used to describe a more universal approach to study the entirety of genes (genomics), mRNA transcripts (transcriptomics), proteins (proteomics) or metabolites (metabolomics) of a sample, cell or organism (see **Figure 1.2**). A vast amount of data becomes available with these technologies and techniques become faster and cheaper. Sections 1.4.3.1 to 1.4.3.5 will summarise the strength and limitations of each “-omics” approach. Further described is the information gained so far from studies using these methods to investigate JSLE.

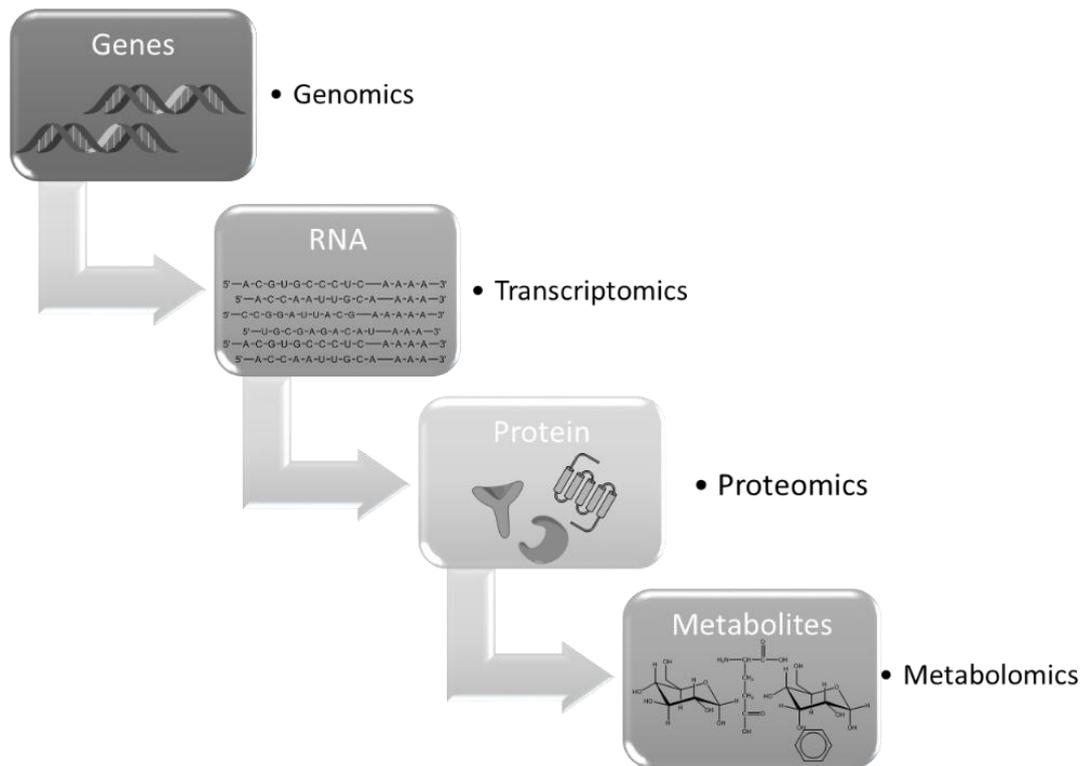


Figure 1.2: Omics approaches can investigate the gene level (genomics), RNA level (transcriptomics), protein level (proteomics) or metabolite level (metabolomics) of a sample.

1.4.3.1 Genomics and JSLE pathogenesis

The genome is the genetic library coding for all transcripts and therefore proteins. Single nucleotide polymorphisms (SNP) in a gene can cause the resulting protein to be overactive, inhibited or even dysfunctional. SNPs and copy number variants (CNV) can give information about cell functions that are likely to be perturbed in a disease. While SNPs can alter the function of proteins, CNVs can change the amount of protein present.

Different cellular actions have been indicated to play a crucial role in the immunopathogenesis of JSLE based on genomic observations. These include: abnormalities in apoptosis, IFN-signalling, phagocytosis, complement system, B-cell signalling, T-cell signalling, or DNA-processing. Examples of these are given in **Table 1.3**.

CELL FUNCTION EXAMPLES FOR GENES SHOWN TO BE ALTERED IN SLE PATHOGENESIS (REFERENCE)

APOPTOSIS	ATG5, FAS, FASL [69], [70]
INTERFERON-SIGNALLING	IRF8, IFIH1 [71]
PHAGOCYTOSIS	FCGRIIA, FCGRIIIA [72]
COMPLEMENT SYSTEM	C4A, C4B [40], [41]
B-CELL SIGNALLING	BANK1, BLK [69], [71]
T-CELL SIGNALLING	CTLA4, PTPN22 [73], [74]
DNA-PROCESSING	DNASE1, TREX1[42], [75]

Table 1.3: Different functions are suggested to be altered on the genome level in SLE.

Nevertheless, despite CNV of genes or SNPs, transcription of these genes may be regulated via other genes and the functionality of the proteins they express needs to be assessed carefully before conclusions about the effect of them in any given disease can be made. Alterations in the genome may increase or decrease susceptibility but are not in themselves definite predictors that disease will occur during an individual's life. Thus, knowledge derived from genomics has to be understood for what it can and cannot reveal and is not absolute; its interpretation needs to be treated with care.

1.4.3.2 Transcriptomics and JSLE pathogenesis

RNA is the transcript of the genomic information and can mirror need of a specific tissue, the response to the environment, or a specific physiological condition. Transcriptomic analysis can be achieved by a range of techniques

including microarray which uses a selected range of sequences or RNA-sequencing (RNA-seq) which analyses all transcripts.

Transcriptomics data in patients with adult-onset SLE and JSLE has identified clusters of genes expressed specifically, that indicate an increased IGS and the importance of neutrophils in the disease process (as described in 1.4.2).

1.4.3.3 Proteomics and JSLE pathogenesis

Transcripts can be degraded quickly or may be available longer for translation which is the process of protein synthesis arising from mRNA. Transcriptomics alone can therefore only give an indication of the state of a cell, but may not actually affect the cell or tissue if it is not translated into protein [76]. The function of many of the proteins expressed from the genes that comprise the IGS is also unclear.

Hence, proteomics research can only detect the presence of a protein and not its function or activity. This approach is often used to identify biomarkers for both SLE as well as JSLE. In JSLE, urinary biomarkers have been found with this approach to identify renal involvement and active nephritis [77]. Several other studies have found biomarkers to predict active or inactive LN [78], [79]. While proteomics data could distinguish JSLE patients with nephritis from JSLE patients without, and active from inactive renal disease, biomarker peak intensities were not able to predict different subtypes of nephritis in JSLE patients [77]. Other studies found good sensitivity and specificity for the prediction of the class of nephritis if proteomics data was fed into an artificial network. The only exception was class V, which is the membranous form of LN [80]. Urine samples are less invasive and therefore preferred for biomarker search using proteomics. Nevertheless, organ damage and kidney involvement has also been assessed with plasma of SLE patients in a Czech cohort [81]. Most papers focus on biomarker discovery rather than further using this tool for exploration of SLE pathogenesis. Nevertheless, these discoveries have been very useful to patients as panels of proteins have been developed to identify nephritis in JSLE [82] as well as remission and flares in patients [83].

While protein expression can help inform what a cell or tissue is expressing, it is still not guaranteed that those proteins are having the specific function they are expected to, as an enzyme or a receptor's activity can depend (for example) on the presence of inhibitors or activators.

1.4.3.4 Metabolomics

The study of metabolites, called metabolomics takes a very different approach to this problem, as it is focused on side and end products present in a fluid, tissue or specific cell type. They are the result of “reactions that have happened” rather than “reactions that can potentially happen” as it is the case in genomics, transcriptomics and proteomics. Despite this, it has to be considered though, that observations can be a result of accumulation of a continuous process, as well as a response to an immediate event.

Two techniques are currently used in metabolomics to quantify metabolites: mass-spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Similarities and differences between these techniques are shown in **Table 1.4** [84].

	Mass spectrometry	NMR spectroscopy
Processing of samples	More time, destructive (ionization)	Little time, non-destructive
Detection	Targeted, predefined	Untargeted, unbiased
Sensitivity	High sensitivity, but potential loss of metabolites in ionization step	Only metabolites with higher abundance
Advantages	Identifies specific metabolites	Analysis of metabolic pathways, cost-effective, high-throughput possible

Table 1.4: Differences between mass spectrometry and NMR spectroscopy as used for metabolomics.

While in MS the sample needs to be ionized first and is then detected according to its mass-to-charge ratio, samples used for analysis with NMR spectroscopy remain intact. For the latter, only a magnetic field is applied to the sample and it gets excited with radio frequencies. The resonance of the nucleus is then measured and noted as a chemical shift for each atom in parts per million (ppm). The chemical environment, which stands for the shielding against the magnetic field, defines the ppm of a nucleus. This can be influenced by electronegativity, hydrophobic interactions or chemical bonds. For example, alcohols are found at lower ppm compared to aldehydes or aromatic rings (Figure 1.3).

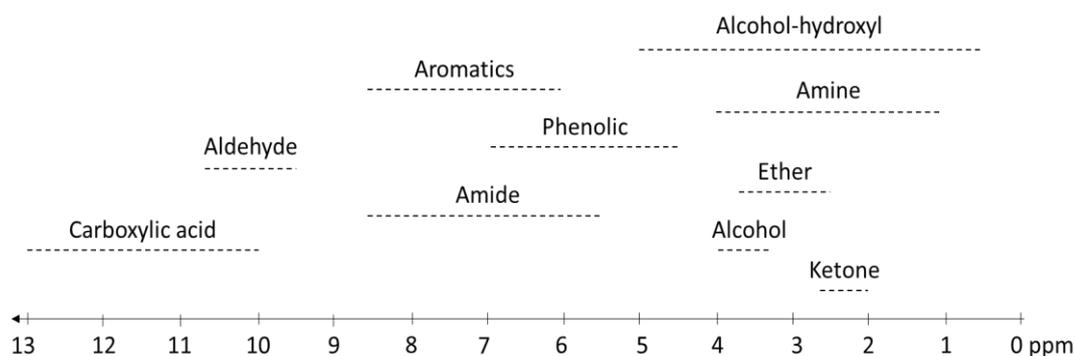


Figure 1.3: Overview (simplified) of chemical shifts. Different chemical groups are shown according to their chemical shifts in ppm. While for example amino acids such as alanine are found between 1-4 ppm, carboxylic acids are found on the far right towards 10-13 ppm.

Nevertheless, even when metabolites are expressed as ppm, these are just a relative value as they are noted as a distance from a reference value and can therefore be different for the same atom measured with another reference compound. Only nuclei with an uneven number of nucleons which consists of neutrons and protons can be detected. Detection is restricted to these atoms with a so called $\frac{1}{2}$ spin as for example ^1H , ^{13}C or ^{14}N . The most abundant one in nature is ^1H and it consequently is a more frequently measured atom.

1.4.3.5 Metabolomics of SLE

Both MS and NMR have been used to a small extent to date to investigate SLE metabolite profiles. Predictive models for LN have been developed on NMR-derived metabolomics data in which it has been suggested that nephritis can be distinguished from patients without renal involvement [85]. Spectra from urine samples can be further analysed to differentiate between specific sub-classes of LN [85], [86]. There are studies testing predictive models of metabolites to distinguish SLE patients from healthy controls or other diseases [87], but investigation of SLE pathogenesis with metabolomic approaches are neglected. Further, most studies focus on serum or plasma metabolites even though urine can be obtained in a less invasive manner. Urine is rather used in studies focusing on kidney involvement.

Importantly, no studies to date have been published that have investigated metabolite profiles in JSLE.

1.4.4 Potential causes for autoimmune development in JSLE

1.4.4.1 Potential role of B and T-cells in JSLE

T- and B-cells are part of the adaptive immune system and possess antigen specific receptors. Specialised antigen-presenting cells like dendritic cells (DC) or macrophages present exogenous proteins on MHC-II molecules and all cells within the body can present endogenous antigens on their surface with MHC-I molecules. This is followed by activation of T-cells once they meet the cell presenting a protein they recognize with their T-cell receptor (TCR). T-cells which recognize self-antigens should normally be removed in a selection process and remaining auto-recognizing cells can cause autoimmunity [88].

T-cells comprise a number of subtypes, and depending on the specific T-cell subtype they have different roles and react with a specific response. One type are, CD8⁺ cytotoxic T-cells which can directly kill infected or damaged cells by releasing granzyme B and perforin which mediate apoptosis of the target [89]. Cells present endogenous protein on the MHC-I on the cell surface and can thereby signal to the cytotoxic T-cells if they need to be eliminated.

Autoantigens should therefore be ignored by this type of T-cell. Even though information is scarce in autoimmunity, in SLE, cytotoxic T-cells have been found in higher proportion in blood of active patients compared to patients with inactive disease. The percentage of cytotoxic granzyme B and perforin positive cells correlated with the SLEDAI of the patients [90].

T-helper cells are another type of T-cells which regulate other immune responses with differing patterns of cytokine production. Regulatory T-cells (T_{reg}) have a role in dampening an immune response and produce transforming growth factor β (TGF- β) and IL-10. Proportion of these cells was reduced in active SLE patients compared to inactive patients and T_{regs} of SLE patients were more sensitive to Fas, a protein inducing apoptosis [91]. Increased sensitivity to apoptosis has not been observed for cutaneous lupus, but a decreased proportion of T_{regs} was detected in skin biopsies from cutaneous lupus patients compared to healthy control samples [92].

Th1, Th2 and Th17 cells trigger further responses. Th1-cells produce IFN γ and IL-2 for the defence against microbes, whereas Th2-cells produce IL-4, IL-5 and IL-13 and fight helminths. Th17-cells cause an inflammatory response with the production of IL-17 and IL-22 [93], [94]. Th17 cells especially cause inflammation and impaired IL-2 production in SLE patients [95] may skew T-cells to rather develop into inflammatory Th17 cells instead of T_{regs} [95]. Th17-cells were further discussed for JIA (see Section 1.1.2) and may cause similar damage in JSLE.

B-cells recognize antigens via a B-cell receptor (BCR) similar to the TCR and produce antibodies which protect the body from recurrent infections. After activation class switching ensures that different classes of antibodies can be produced such as IgE, IgA, IgM and IgG [96]. Production can be stimulated both with and without Th cell assistance [97]. During development B-cells undergo a selection process and those with high-affinity for autoantigens undergo apoptosis [98]. Dysfunctional selection increases the number of autoreactive B-cells present and is found to be a problem in SLE and may explain autoreactive antibodies [99]. SLE is indeed characterised by the presence of autoantibodies, indicating a major importance of B-cell dysfunction in the pathogenesis of SLE

[100]. Survival of autoreactive B-cells may be supported by proteins stimulating proliferation of B-cells, such as BAFF. BAFF inhibits apoptosis and has been found to be significantly increased in SLE patients [45].

B- and T-cells, together referred to as lymphocytes, trigger production of anti-nuclear autoantibodies such as anti-chromatin/nucleosome or anti-dsDNA antibodies in autoimmunity. However, the origin of the antigens that cause antibody production has yet to be fully elucidated [100]. Lymphocytes express increased levels of Fas, also called Fas receptor or apoptosis antigen 1 which induces apoptosis. Higher expression on the cell-surface of SLE lymphocytes compared to healthy control lymphocytes indicates a higher rate of apoptosis and thereby potential presentation of autoantigens. On the other hand the expression of B-cell lymphoma 2 protein (Bcl-2), an anti-apoptotic protein, is upregulated in T-cells of JSLE patients, which might lead to restricted clearance of autoreactive cells [101].

All the aforementioned discoveries of B- and T-cell abnormalities suggest an important role of the adaptive immune system in JSLE.

1.4.4.2 Cytokine abnormalities

Investigation of the underlying pathogenesis of SLE has involved exploration of both the adaptive and to a lesser extent the innate immune system. Communication between all cell types including non-immune cells is achieved with small molecules called cytokines. Many of those are known to be abnormal and dysregulated in SLE patients. Here, the focus will be on three important cytokines relevant to in SLE pathogenesis, namely IFN, TNF α and IL-17.

1.4.4.2.1 Interferons

Interferons are a class of cytokines that have been considered important for protection against viruses [59], but have also been found to play a role in autoimmune diseases. The class of IFNs consist of type I IFNs, which comprise IFN α , β , ϵ , κ , ν , and type II IFN which is restricted to IFN γ , and type III IFNs

consisting of IFN λ . Stimulation of monocyte-derived dendritic cells with lipopolysaccharides (LPS), flu virus or polyinosinic:polycytidylic acid (poly I:C) triggers both type I (α and β) and type III IFN production [102]. Similar to type I IFNs, production of IFN λ activates STAT2 and thereby contributes to antiviral activity and inhibition of cell proliferation [103]. There are not many studies focusing on type III IFNs. Nevertheless, both mRNA and protein expression of type III IFNs have been found to be significantly increased in SLE patients, which included juvenile-onset cases, compared to healthy controls [104]. Furthermore, protein concentration of type III IFNs in serum was significantly higher in active compared to inactive patients. This study demonstrated a strong correlation between IFN λ and anti-dsDNA levels in patients, which could go some way to explaining the increased cytokine release [104].

As already mentioned in Section 1.4.4.1, Th1 cells are producers of IFN γ , the only type II IFN. When IFN γ is injected into lupus-prone mice it stimulates lupus-like glomerulonephritis [105]. Similarly, in IFN γ -transgenic mice a lupus-like phenotype developed with auto-antibodies and nephritis suggesting this IFN to have a higher involvement in the development of kidney disease in SLE [106]. A study investigating IFN γ in SLE patients found increased serum levels of this cytokine and noted that IFN γ levels preceded increased type I IFN activity [107].

Type I IFNs have been investigated much more extensively in SLE to date, and IFN α specifically has been included in a wide range of important studies. This cytokine possesses 13 subtypes and its main producers are plasmacytoid dendritic cells (pDCs) [108]. These cells have been found (among other tissues) in skin samples of SLE patients, especially at the sites where lesions were present, showing their probable involvement in the disease process itself [109]. pDCs produce IFN α , a cytokine important for JSLE (see Section 1.4.2 and 1.4.4.2.1.). Among other stimuli, pDCs release IFN α in the presence of poly I:C, which mimics dsRNA for example of Herpes Simplex virus type I (HSV) or plasmid DNA. A case of an IFN α response that may be relevant to lupus disease was observed after stimulation with apoptotic and necrotic supernatants in the

presence of SLE IgG [110]. A more recent study has suggested that neutrophils are also capable of producing IFN α in response to circulating chromatin. Even though neutrophils may produce less cytokine per cell than pDCs, due to the high abundance of neutrophils in the body, their effect may still exceed the overall effect of pDCs [111]. For some time, IFN α serum levels have been found to be higher for disease active patients. In the first study in 1979 a positive correlation with anti-dsDNA antibodies was also found [112]. In this study, the cytokine was measured indirectly as assays detected antiviral activity and noted this to reflect IFN α levels. Clearly, other factors may have also played a role in antiviral activity, rather than IFN α alone. [112]. Since then, most other studies also focused on indirect measurement of IFN α , for example by looking at IFN-induced genes as described in Section 1.4.2 such as Baechler *et al.* in 2003 [54]. In SLE nephritis biopsies, mRNA staining of IFN α was positive in renal epithelial tubular cells, suggesting that these cells can also produce the cytokine [113]. IFN α has been shown to be present in SLE serum, but to be even higher in JSLE patients. Cytokine levels were shown to correlate with IGS for SLE, JSLE and other diseases, while JSLE patients had higher IFN α levels than SLE patients [114]. Of note, in this study, one of the adult-onset SLE patients had an IFN high signature with low IFN α levels and another one had an IFN low signature with high IFN α levels which could not be explained. IFN α levels above 300 fg/ml were further associated with high disease activity [114]. Discrepancies between IFN α levels and the IFN signature could be explained by mutations in genes known to cause an IFN signature [57], or could indicate the influence of other cytokines.

Most studies described above focus on adult-onset SLE leaving childhood-onset under-investigated in comparison. Furthermore, the impact on disease pathogenesis of patients dependent on whether they are IFN high or IFN low has not been investigated and may be essential for treatment of JSLE.

1.4.4.2.2 Tumour necrosis factor-alpha (TNF α)

TNF α is an important cytokine, considered to be proinflammatory, with multiple functions including apoptotic and proliferative effects [115], [116]. On one hand, TNF α can help trigger apoptosis [116] when acting through TNF-receptor 1 (TNFR1) via the Fas-associated protein with death domain (FADD) [117]. On the other hand TNF α is proposed to have anti-apoptotic effect [118] when signalling through TNFR2 [119]. Furthermore, TNF α treatment of T-cells has caused them to respond with stronger proliferation [120]. Similarly, B-cells require TNF α to proliferate and in the presence of TNF α enhanced proliferation is observed [121].

A link between SNPs in the TNF α gene at position 308 and SLE and JSLE was observed, although this has not been explored further if this has an effect on the protein expression [122], [123].

The pro-inflammatory effect of TNF α in SLE pathogenesis is controversial as some studies suggest its importance and others indicate it may have no influence on the disease process. A positive correlation has been noted between the SLEDAI score of SLE patients in an Egyptian patient cohort and serum TNF α concentration [124]. In contrast a different study measured high levels of TNF α in disease inactive patients [125]. Lower disease activity was further marked by higher TNF α concentrations than high disease activity [125]. Nevertheless, both studies found higher levels of this cytokine in SLE patients compared to healthy controls.

Use of anti-TNF α antibodies in the treatment of SLE is controversial. These agents, such as infliximab, have been shown to ameliorate manifestations including arthritis and nephritis in both SLE and JSLE patients, but results were not stratified according to the age of onset [126]. Interestingly, serum levels of TNF α had more than doubled following six months of antibody treatment, although this did not differ significantly between those treated with conventional therapy and the infliximab-treated group [126].

Importantly though, it has also been observed that anti-TNF α treatment of arthritis patients with adalimumab, etanercept or infliximab has led to induction of lupus with cutaneous manifestation, fever, polysynovitis or even nephritis. The underlying diseases associated with arthritis were RA, psoriatic arthritis, JIA, Crohn's disease ankylosing spondylitis. The study design related to each of these reports was unable to determine if the antibody treatment was the sole cause for lupus-related symptoms [127]. Animal models have shown that absence of TNF α can enhance SLE development. One study for example knocked out the TNF α gene in mice otherwise not susceptible to lupus and found them to develop disease similar to an established LN mouse model [128].

1.4.4.2.3 Interleukin-17 (IL-17)

The family of IL-17 cytokines includes a number of sub-types, namely IL-17A, -17B, -17C, -17D, -17E and -17F. However, the IL-17A form is most commonly referred to as IL-17 and will be discussed specifically here.

IL-17 is produced generally by Th-17 cells and induces release of granulocyte-colony stimulating factor (G-CSF) and IL-8 and thereby attracts neutrophils and stimulates their development [129]. Further, IL-17 together with TNF α has been suggested to promote a pro-inflammatory and pro-apoptotic environment [130].

As described in Section 1.1.2, IL-17 has been studied in JIA and is considered a main driver of the disease. In freshly isolated T-cells from SLE patients, the production of IL-17 has been found to be increased [131]. These cells were detected in the kidney biopsies of SLE patients suggesting attraction of neutrophils into the kidney and giving potential explanations for damage and induction of LN [131].

IL-17 has been shown to correlate with a patient's SLEDAI score in a group of SLE patients with an age range from 18-40. They were described as new-onset patients, but as diagnosis can take time this population may have included some JSLE patients, but this was not specified [132]. Nevertheless, JSLE patients have been described to have higher levels of IL-17A in plasma and in supernatant of

PBMCs stimulated with CD3/CD28 *in vitro*. However, IL-17A was higher in only a minority of JSLE patients than in healthy controls [133].

1.4.4.3 Potential role of neutrophils in JSLE pathogenesis

Many reports indicate the innate immune system to be especially involved in the initiation of SLE pathogenesis, and to be a potential source for the nuclear autoantigens so characteristic of the disease.

1.4.4.3.1 Neutrophils and their natural history

One essential cell of the innate immune system is the neutrophil, a granulocyte which is the most abundant white blood cell or leukocyte in the blood. In response to infections they are the first actors in the defence machinery of the immune system. They were originally considered to only have a half-time in the blood of between 4-9 hours and a high turnover rate of $86-341 \times 10^7/\text{kg}/\text{day}$; they then are likely to die or migrate into tissue. In healthy individuals, both their half-time in the blood and turnover of neutrophils exhibits significant variability [134]. Their lifespan is still under discussion as a more recent study proposed that neutrophils have a half-life of 5.4 days in the peripheral blood. This study considered that the handling of the neutrophils may have an important impact on their perceived lifespan and the presumption of their rapid death was actually just an observation caused by an artefact of the original assay [135]. At the end of their life, neutrophils upregulate CXCR4 and migrate to the bone marrow where they undergo apoptosis [136]. This function appears to be dysregulated as neutrophil apoptosis in the peripheral blood is increased in JSLE [137]. The role of cell death especially of neutrophils in SLE is further discussed in Section 1.4.4.4.

1.4.4.3.2 Neutrophil function

As first guards of the immune system, neutrophils use several means to fight infections. These include phagocytosis (further discussed in Section 1.4.4.5),

meaning engulfing the pathogen and killing it with reactive oxygen species (ROS), degranulation, meaning releasing their toxic granules and NETosis (further discussed in Section 1.4.4.4.2), a special cell death that involves extruding their chromatin.

Upon infection neutrophils get attracted to the site of infection, for example by a complement component such as C5, cytokines such as IL-8 or bacterial components such as N-Formylmethionyl-leucyl-phenylalanine (fMLP) [138]–[140]. Reaching the targets they then detect them either directly via Toll-like-receptors (TLRs) which recognize pathogen-associated molecular patterns (PAMPs) (more details found in Sections 1.4.4.5 and 1.4.5.1) or via molecules which opsonize the pathogen (further described in Sections 1.4.5.3 and 1.4.5.3). ROS production in the phagosome via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase supports the killing of the pathogen inside the cell and dysfunction of this enzyme can lead to a serious disease called chronic granulomatous disease [141]. The killing of pathogens further requires degranulation, meaning release of the enzymes and peptides from the preformed granules into the phagosome [142]. Neutrophil granules are divided into primary (azurophilic), secondary (specific) and tertiary types. Myeloperoxidase (MPO), cathepsins, elastase and defensins are found in the primary granules which are the most toxic ones. Secondary granules contain lactoferrin as the main protein and tertiary granules contain gelatinase [143]. NETosis occurs when pathogens are too big to be phagocytosed. As proteins from primary granules can be released from the cells via NETosis they are considered to cause harm to the surrounding tissue in diseases like RA and SLE [144], [145]. Furthermore, antibodies against MPO have been found in SLE demonstrating the role of neutrophils in disease development [146].

1.4.4.4 Cell death

Depending on the stimulation and predisposing factors, there are different ways for a cell to undergo cell death. Also called a programmed cell death, apoptosis can be induced both from within e.g. by stress or DNA damage, but also from

outside the cells via the Fas-receptors. The aim of apoptosis is to clear cells in a non-inflammatory manner which makes it suitable for processes like tissue development or homeostasis. One of the key steps of apoptotic cell death is the condensation and fragmentation of chromatin, which might be a way of preventing immune responses [147]. Apoptotic cells release signals and present them on their cell surface, such as phosphatidylserine. This way they get cleared away and even though many cells undergo apoptosis every day only few are found in tissues or in circulation [148].

If apoptotic cells are not removed they start losing their membrane integrity leading to secondary necrosis. Direct induction of necrosis is possible, and both the cellular organs and cytoplasm swell until the cell membrane ruptures. This occurs if there is no chance for repair after significant cell damage and leads to an uncontrolled death. Over the last few years it has become clear that there is also programmed necrosis induced for example by $\text{TNF}\alpha$. These pathways are further divided depending on their intracellular signalling into necroptosis, mitochondrial permeability transition-regulated necrosis, and parthanatos [149]. Oxidative (also called) respiratory burst has been observed for both primary necrosis and secondary necrosis meaning that there is a release of ROS [150]. The rupture of the necrosing cells further releases larger molecules into the environment such as damage-associated molecular patterns (DAMP) and DNA [151].

1.4.4.4.1 Apoptosis in JSLE

Among the different types of cell death described (see Section 1.4.4.4), apoptosis has been found to be dysregulated in both T- and B-cells in JSLE [101]. Modifications of the chromatin, like citrullination or acetylation, occur during the programmed cell death and make DNA in combination with other molecules like High-mobility group box-1 protein (HMGB1) immunogenic. As long as apoptotic cells get removed the process is invisible to the immune system, but if they remain for longer, they become necrotic. Then the modified chromatin with

HMGB1 is available to dendritic cells (DC) for uptake, which can trigger auto-antibody production [152].

Autoantibodies in lupus can be directed against SS-A/Ro an extractable nuclear antigen (ENA) and Smith antigen (Sm). These proteins stay intact and are not cleaved by proteolysis during apoptosis or primary and secondary necrosis [153]. They therefore serve as ideal targets for the immune system to develop autoantibodies. More apoptotic cells are observed in SLE patients compared to healthy adults; an increase in apoptosis, a faulty signalling towards phagocytic cells, or dysregulation of phagocytosis could be a reason [148]. In JSLE, neutrophils themselves show a higher rate of apoptosis, and additionally the serum of these patients can promote apoptosis compared to serum from non-lupus patients. Both Fas Ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) which are known to be pro-apoptotic factors are found to be elevated in JSLE serum [137].

1.4.4.4.2 Neutrophils and the Neutrophil Extracellular Traps (NETs)

Neutrophils are part of the innate immune system and release as part of their defence cytotoxic contents. This serves as an effective protection against pathogens but can also cause tissue injury leading to non-infectious inflammatory diseases. 50-60% of patients with SLE develop neutropenia suggesting an important role for neutrophils in this disease [137]. Neutrophils are an abundant cell type and as they have a short life-span they may already be an apoptotic burden to the immune system. If control of neutrophil apoptosis or clearance is dysregulated, this may lead to the presentation of autoantigens to the immune system and may support development of autoimmune diseases [154]. One of the roles of neutrophils is clearance of pathogens by phagocytosis (further explained Section 1.4.4.5), but this process can be insufficient for the number of pathogens when they replicate too quickly or if the pathogen is too large to be taken up.

In these circumstances, the release of neutrophil extracellular traps (NETs) in a process called NETosis is required, which is a cell type specific death that only

occurs in neutrophils. Activated platelets, bacteria, and also fungi can trigger this release of chromatin with antimicrobial, granule specific proteins attached. This defence mechanism involves perforation of the cell membrane and thereby the death of the neutrophils [155]. Upon stimulation, neutrophil elastase (NE), an enzyme usually located in the granules, transports into the nucleus where it is responsible for the degradation of the chromatin. Myeloperoxidase then breaks down the nuclear envelope and further processes the chromatin. After about 120 minutes the cell membrane ruptures and NETs are released to capture the pathogen. It should be noted that recent data also suggest that cell survival after NET release is possible [156].

1.4.4.3 NETosis in JSLE

Besides apoptosis, NETosis also seems to be of great importance in the aetiopathogenesis of JSLE. As in programmed cell death (apoptosis), in NETosis chromatin also undergoes modifications but decondensation is necessary before it can be released into the extracellular environment. NETs can therefore become a source of auto-antigens and subsequent targets for immune complexes and complement. NETs can also cause direct tissue damage and can therefore be involved both in the development of the disease and its symptoms [157]. Increased NETosis and impaired NET removal seems to be involved in SLE as NETs have been found in SLE both in blood and in tissues [158]. Very recently our group has demonstrated that a subset of neutrophils, the so-called low-density granulocytes, is augmented in JSLE patients [159]. This cell type displays inflammatory capacity and is more likely to produce NETs [159]. Dysregulated apoptosis and NETosis might explain why more apoptotic cells and NETs are seen in JSLE patients. This apoptotic and inflammatory environment in turn also affect cells in patients and causes them to be dysregulated.

1.4.4.5 Phagocytosis

Phagocytosis is a process of removing bacteria, debris and apoptotic material safely. Cells responsible for the removal of apoptotic cells or pathogens are called phagocytic cells. They can be divided into professional phagocytes, which are macrophages, monocytes, immature dendritic cells and neutrophils, and non-professional phagocytes like NK-cells, lymphocytes, and surrounding cells. To be removed the apoptotic cells send “find/eat me” signals to professional phagocytes. One example is sphingosine-1-phosphate which is released after stimulation of apoptosis and which then attracts cells such as monocytes or macrophages [160]. Another example of an attractant of professional phagocytes are nucleotides present in apoptotic supernatants with ATP or UTP being stronger attractants than ADP and UDP [161]. In addition, apoptotic cells can release signals to keep cells which would cause inflammation away. For example, neutrophil attraction could cause inflammation and it could therefore be beneficial for apoptosing cells to attract only monocytes/macrophages for removal. The presence of lactoferrin will repress chemotaxis only of neutrophils even in the presence of strong neutrophil-chemoattractants like IL-8 or fMLP [162]. When reaching the apoptotic cells further “eat me” signals such as from phospholipid phosphatidylserine on the cell surface are required to start the engulfment by the phagocytes [163].

Microorganisms on the other hand are recognized via PAMPs with pattern recognition receptors (PRR). Patterns on bacteria include cell wall components such as LPS, peptidoglycan or lipoproteins. Fungi possess components such as mannans, glucans, zymosan or chitins that can be recognized. The immune system possesses proteins which can detect these patterns. These include Toll-like receptors (TLRs), lectins, nucleotide oligomerization domain proteins (NOD) or peptidoglycan receptors [164]–[166].

After engulfment phagocytes can either retain bacteria intracellularly to stop them from proliferating, which is a state called bacteriostasis, or can digest and thereby kill them. Digestion happens within the phagosome when fusing with the lysosome by acidification and breakdown by enzymes [167].

1.4.4.5.1 *Phagocytosis in JSLE*

To date, there is a paucity of robust data available describing the phagocytic activity and control mechanisms in JSLE, even though dysregulation of phagocytosis can cause presentation of nuclear antigens.

PBMCs, for example, provide evidence of reduced clearing of apoptotic material in *in vitro* experiments [168]. As the study of Ballantine *et al.* 2015 showed, serum from JSLE patients decreases phagocytosis of *E.coli* and apoptotic neutrophils by healthy macrophages, whereas monocytes from healthy individuals retained normal phagocytosis function when they were incubated with their own serum. The phagocytic ability of JSLE monocytes was also restored when they were incubated with healthy control serum. Tyro3, Axl and Mer were found to be decreased on the cell surface of monocytes and their soluble counterparts to be increased. These molecules are involved in removing apoptotic cells, and by their cleavage off of the surface the ability to phagocytose is diminished. This explains why the monocytes isolated from patients and incubated with patients' serum display decreased capacity to remove bacteria [4]. Further research was undertaken on samples from adult-onset SLE patients. It was shown that their monocytes express less CD44 on their surface than the ones of healthy controls. This molecule seems important for the binding and phagocytosis of apoptotic cells [169]. Despite these findings, the mechanisms behind phagocytosis in JSLE remain poorly understood.

1.4.4.5.2 *Phagocytic clearance by Neutrophils in JSLE*

PBMCs are not the only cells responsible for phagocytosis and neutrophils play at least an equally important role. Reduced phagocytosis has been observed with decreased engulfment of albumin and polyglobin-coated beads in 60% of the adult-onset SLE patients [168]. A similar hypothesis was proposed by Chauhan *et al.* 2015 who investigated NET degradation and phagocytosis. Looking at a heterogeneous group of juvenile- and adult-onset SLE patients, they observed reduced dismantling of the NETs by their serum compared to

healthy controls. When the results were divided into subgroups it was shown that serum of patients with anti-dsDNA and anti-dsDNA plus ENA antibodies showed reduced degradation, whereas samples from patients with just anti-ENA antibodies behaved no differently to healthy controls. Even though there seemed to be a difference in NET degradation between the groups, for all of their serum they saw an effect of reducing phagocytosis in healthy neutrophils [170]. Knowledge about efficacy of phagocytosis and processing of different bacteria by neutrophils of JSLE patients is very limited and the possible dysregulation within the neutrophils is not studied. This underlines the importance of investigating dysregulated phagocytosis in JSLE which may induce NETosis.

1.4.5 Phagocytosis related genes in JSLE patient neutrophils

Dysregulated phagocytosis might not only leave apoptotic debris behind as a source for auto-antibody production, but might also alter the reaction following phagocytic stimuli towards cell death like NETosis [171]. Therefore, phagocytic regulation needs to be investigated. While phagocytic genes have not been investigated as a group, several genomic studies have found phagocytosis related genes to be susceptibility genes. Low copy numbers of C4 were linked to a risk to develop SLE [40] and so were individuals with low copy numbers of FcγRIIIb [172]. Transcriptomic data is rarer and especially neutrophil specific data are lacking.

Transcriptomic data our group acquired from our JSLE cohort showed significant differences between control patients' and JSLE patients' gene expression profiles of neutrophils (unpublished data). For this present study, genes were chosen based on significance in the aforementioned unpublished study and on their involvement in phagocytosis. The functions of these genes are summarized in **Figure 1.4**.

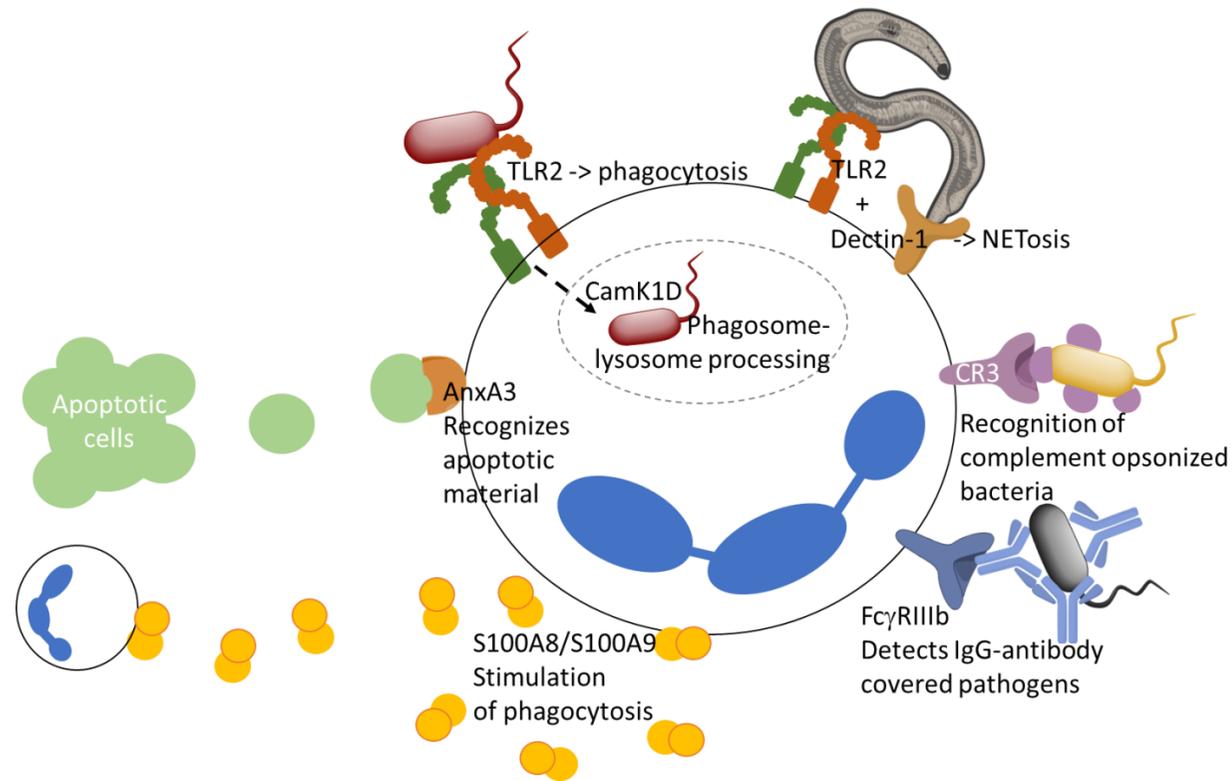


Figure 1.4: Summary of functions of all phagocytosis related genes tested in this thesis. Clockwise, from the left top is shown: TLR2, a pathogen-associated molecular pattern recognition receptor. Dectin-1 recognizing size of pathogens together with TLR2 and leading to NETosis if phagocytosis is not possible. CD3 detects complement opsonized bacteria. Low-affinity FcγRIIIb recognizes antibody covered pathogens. S100A8/S100A9 heterodimer can stimulate phagocytosis in an endocrine and autocrine way. AnxA3 binds to phosphatidylserine of apoptotic cells. Inside the neutrophil: CamK1D, responsible for phagosome-lysosome fusion and ROS production to eliminate pathogens.

1.4.5.1 Toll-like Receptor 2

Toll-like receptor 2 (TLR2) is one of the pattern recognition receptors recognizing PAMPs and DAMPs (described in Sections 1.4.4.4 and 1.4.4.5). This specific receptor forms heterodimers with TLR1, Dectin-1 and TLR6. It is known that lipoprotein is a ligand for TLR2, but the receptor also recognizes zymosan, lipoteichoic acid or LPS. Pathogens containing any of these molecules or patterns, such as bacteria and yeast, will activate the receptor [173]. As for most TLRs, this is followed by recruitment of Myeloid differentiation primary response gene 88 (MyD88) and activation of nuclear factor- κ B (NF- κ B) [174], [175]. Signalling through TLR2 can lead to secretion of both anti- and pro-inflammatory cytokines including IL-10, IL-12, IL-13 and IFN- γ [176], [177]. TLR2 can further localize in intracellular structures and induce type I IFNs and TNF α production [178]. The main function of TLR2 is to create an inflammatory environment through triggering the release of cytokines [179]. It was reported that by adding macrophage-activating lipopeptide 2 (Malp2), which stimulates the TLR2/6 heterodimer, or Pam3CSK4, a synthetic triacylated lipoprotein stimulating TLR2/1, to human neutrophils, they are less likely to undergo apoptosis and showed more phagocytosis of bacteria. In this paper, the effect for Pam3CSK4 was only observed at high concentrations and therefore might not be relevant in physiological conditions [180]. Hellberg *et al.* 2011 also tested the influence of TLR2 on phagocytosis of apoptotic cells. Both Malp2 and Pam3CSK4 incubation resulted in a significant increase of the phagocytic activity of neutrophils and thus demonstrated the link between TLR2 and phagocytosis. The activation by Pam3CSK4 was observed only at high concentrations [181].

1.4.5.2 Dectin-1

Even though TLR2 forms heterodimers with other TLRs, it also interacts with a non-TLR-type receptor. This particular receptor is called Dectin-1 and is a C-type lectin recognizing PAMPs, like β -glucan or zymosan which can be found on fungi [171]. This protein is also known as CLEC7A and is part of the family of non-classical C-type lectins. After ligand binding, the immunoreceptor tyrosine-

based activation motif (ITAM)-like motif of Dectin-1 becomes phosphorylated and activates spleen tyrosine kinase (SYK) [182]. The signalling leads to production of inflammatory cytokines, respiratory burst and phagocytosis [183]. Dectin-1 is important in neutrophils as it can sense the size of microbes and thereby alter the phagocytic process of the neutrophil. Fungi for example can be present both in small yeast but also in a large filamentous hyphae form, and it is important to distinguish between these states. While the yeast can still be phagocytosed, the size of hyphae triggers NETosis. Similar observations have been made with bacteria that form larger aggregations and therefore inhibited phagocytosis. Branzk *et al.* 2014 suggest that this discrimination and the following reaction of the cell is dependent on Dectin-1, as its deficiency leads to NET release in response to a pathogen independent of the size of the microbe [171]. Thus Dectin-1 is essential for phagocytosis and it has been found to be both defective and associated with fewer monocytes in SLE and rheumatoid arthritis compared to healthy controls. In active disease a significantly lower Dectin-1 protein expression has been found than for inactive SLE [184].

1.4.5.3 Complement receptor 3

Both CD18 and CD11b are integrins and are the two subunits that make up complement receptor (CR) CR3. This heterodimer can bind to iC3b which opsonizes apoptotic cells. Stimulation of CR3 can alter cytokine production and phagocytosis so that the apoptotic cells are cleared [185]. Links between CR3 and SLE have been observed, as mutations in the gene for this protein are highly associated with disease development. Some of the mutations studied were also tested for their functionality and revealed impaired phagocytosis [186], [187].

1.4.5.4 Fc γ RIIIb

IgM, IgA, IgE and IgG antibodies all have their specific receptors which are required to detect antibody-opsonized targets. The Fc γ receptor IIIb also known as CD16, is a protein which binds the constant region of IgG with low-affinity. CD16 can be found on other cells (CD16a), but CD16b is considered exclusive to

neutrophils linked to the cell membrane via glycosyl-phosphatidylinositol [188]. Each neutrophil possesses between 120,000 and 400,000 molecules on its surface [189]. The Fc γ receptor has been detected intracellularly from compartments which can quickly restore cell surface protein [190]. This is necessary, because besides the location on the cell surface and intracellularly, CD16b can be shed. Cleavage into the supernatant or blood from the surface requires ADAM metallopeptidase domain 17 [191]. This is caused for example by actin polymerization or cell death [192], [193].

Neutrophils are able to bind to IgG complexes and this process has been shown to be linked to CD16b as its blockade inhibited binding of complexes [189]. Targeting CD16 with antibodies also decreased phagocytosis of heat-killed opsonized *S.aureus* by neutrophils [194]. Fossati *et. al* supported the theory further when they depleted this protein. They demonstrated that a lack of Fc γ RIIIb leads to a decrease both in phagocytosis of IgG-opsonized beads and in activation of the respiratory burst, which are both essential for efficient phagocytosis [195].

1.4.5.5 S100A8 and S100A9

The S100A9 and S100A8 proteins form heterodimers called calprotectin and upon Ca²⁺ binding they create a heterotetramer. Their expression in keratinocytes and epithelial cells is induced by inflammation, but they are found even under healthy conditions in monocytes and neutrophils. Activated phagocytes need the heterotetramer for their migration as it is part of the cross-linking of the microtubules [196]. Calprotectin and S100A9 alone also stimulate the NADPH oxidase complex which is linked to the phagosome. That is probably why a knockdown of S100A8/A9 results in a decrease of phagosomal ROS-production [197]. Simard *et al.* further investigated the influence of S100A9 on phagocytosis and found neutrophils to have stronger Fc γ R- and CR-mediated uptake when S100A9 was added to the neutrophil medium. Upon activation with S100A9 neutrophils phosphorylate Syk, a kinase implied in phagocytosis. A cell can stimulate itself in an autocrine manner with S100A9 and thereby be

more prone to perform phagocytosis. This involves S100A9 in the process of phagocytosis [198].

1.4.5.6 Annexin A3 (AnxA3)

The annexin family consists of proteins that are regulated by Ca²⁺-signalling and they are thought to be involved in exocytosis (transport out of the cell) and endocytosis (transport into the cell) [199]. Very little is known about the annexin family and even less is published about AnxA3. For Annexin A1 and A2 it has been reported that they bind to S100 proteins and, besides trafficking, the protein family is also implicated in inflammation and apoptosis [200].

AnxA3 is found in cytoplasmic granules in resting neutrophils. When the cells are activated upon phagocytosis of opsonized zymosan, the protein localizes at the membrane of the phagosome instead [201]. Rosenbaum *et al.* demonstrated that mouse AnxA3 can interact with phosphatidylserine, which is presented by apoptotic cells. Binding of AnxA3 was shown on both early apoptotic, and secondary necrotic cells. This suggests an important function in the clearance of dying cells as annexins are found both on apoptotic cells and phagocytes [202]. Even though the study was performed with mouse AnxA3, the genetic differences with the human equivalent have been proposed to not result in differences in function [203].

1.4.5.7 CamK1D

Ca²⁺/Calmodulin-dependent kinases are responsible for a downstream signalling and include the enzymes CamK1-4. For activation they either have to be phosphorylated or the auto-inhibitory domain in the protein has to be removed [204]. There are four different isoforms of CamK1, namely CamK1 α , β , γ and δ . A synonym for CamK1 δ (CamK1D) is CamK1-like kinase and it is also called CKLiK. The mRNA of CamK1D is found in granulocytes but is hardly expressed in monocytes and lymphocytes [205].

Ca²⁺ levels change upon activation of neutrophils and Ca²⁺ being important for respiratory burst, phagosome-lysosome fusion and adhesion, suggests a role of CamK1D in these processes. For phagocytosis to be properly functional both adherence to the pathogen and the production of ROS, as well as the fusion of the phagosome with the lysosome, are essential. It has been shown that CamK1D can activate ERK1, a kinase regulating phagocytosis [205], [206]. In 2005 the same group showed that by inhibiting the kinase function of CamK1D, ROS production and phagocytosis were reduced [207].

1.4.6 Summary

Autoimmune diseases are conditions which affect patients' lives in a very serious way. The impact of autoimmune disease is even greater for children who exhibit symptomology and disease burden over a longer life course, particularly as contemporary disease management offers a longer life expectancy. Furthermore, autoimmune disease often differs in children in important features and clinical characteristics, who cannot just be seen as a small adult. The specific needs of children and young people with autoimmune diseases such as JSLE and JIA need to be directly addressed. There is much to learn regarding the aetiopathogenesis of SLE from adult patients, but to date, less is known regarding the specific characteristics in JSLE patients. Stratifying patients into both juvenile-onset and adult-onset disease offers opportunities to learn specific aspects of disease mechanism in this wide spectrum of disease. While there have been several approaches to study these diseases to date including genetic, transcriptomic data and proteomic data, the contribution of metabolomic data to this process are still lacking, especially in paediatric autoimmune diseases including JSLE and JIA.

In recent years there has been considerable attention given to exploring and understanding the role of the IGS in the aetiopathogenesis of JSLE, and in particular whether it can be another way of stratifying patients. Specific consideration has also focussed on the IGS and the importance of neutrophils in the evolving disease mechanisms. Patients can be divided into both IFN high and

IFN low expressing subgroups. However, to date, there have been no specific investigations undertaken to compare the metabolite profiles of these patient groups stratified by IGS expression.

Within the different IGS patient subgroups, a phagocytosis-related gene signature (PRG signature, PGS) has also been detected in the transcriptomics data. Therefore, it can be hypothesised that phagocytosis is dysregulated which may lead to increased NETosis and that this process may be considered a potential cause for autoantibody production in JSLE patients. Understanding the role of these specific characteristics of neutrophil function and their involvement in the evolving disease mechanism of this archetypal autoimmune disease is therefore important. Several factors inhibiting phagocytosis in both adult-onset SLE and JSLE patients have been described, but a direct investigation of the ability of JSLE neutrophils to phagocytose has yet to be performed. Investigation to date of the triggers and causes of the PGS, and the IGS in JSLE, is very limited.

1.4.6.1 Overarching hypothesis

It is possible to stratify JSLE patients based on their metabolite profiles present in serum and urine or by phagocytic and IFN-induced gene signatures present in neutrophils from whole blood. The PGS should be visible in the phagocytic activity of PMN of JSLE patients and signatures are caused by the environment of PMN.

1.4.6.2 Overarching aims

Aim 1: To explore the ability to stratify patients with autoimmune diseases based on their IGS, including a metabolomics approach using urine and serum.

Aim 2: To investigate the PRG and IG signatures in neutrophils of JSLE and healthy paediatric controls.

Aim 3: To determine if the PGS is translated onto protein and functional level in neutrophils of JSLE patients.

Aim 4: To determine the influence of factors present in the neutrophil environment including $\text{TNF}\alpha$, $\text{IFN}\alpha$, nucleosomes and signal released from apoptosing cells, on the PGS.

2 Materials and Methods

This chapter describes materials and general methods used to achieve the aims stated in Section 1.8.2. At the beginning of Chapters 3-5 the methods specific for each chapter are described.

2.1 Patients and sample collection

2.1.1 Patient criteria

Urine and whole blood samples were collected from patients who were part of the UK JSLE Cohort Study and Repository. The study was approved by the North West Liverpool East Research Ethics Committee (REC: 6/Q1502/77) (Appendix A 1). JSLE patients were diagnosed according to the revised American College of Rheumatology (ACR) criteria for SLE [27] before the age of 17 years. Patients were recruited from those attending the Department of Paediatric Rheumatology, Alder Hey children's NHS Foundation Trust, Liverpool, UK. Whole blood and urine samples were collected with the support of the National Institute for Health Research (NIHR) Alder Hey Clinical Research Facility. Additional whole blood samples were received from the Central Manchester University Hospitals NHS Foundation Trust from the Royal Manchester Children's Hospital.

JIA patients met the criteria for the International League of Arthritis and Rheumatism (ILAR) classification [5]. Samples from these patients were also recruited from the Department of Paediatric Rheumatology at Alder Hey, and collected with the support of the NIHR Alder Hey Clinical Research Facility.

Paediatric non-inflammatory controls were defined as children attending Alder Hey NHS Foundation Trust for elective surgery where no inter-current infection was present, and with no history of paediatric autoimmune disorder.

All patients and their parents were given detailed information regarding participation in the study (see Appendix A 3.3.3.1 and 3.3.3 for an example). Written informed patient/parental assent/consent was obtained in accordance with the declaration of Helsinki (see Appendix A 3.3.2, 3.3.4, 3.3.5, 3.3.7, 3.3.9 and 3.3.10).

Volunteers for healthy adult control blood declared themselves as healthy and were aged over 18 years. These samples were used for specific experiments when higher cell numbers were needed than were obtainable from paediatric controls. Healthy adult controls were recruited from staff and students of the University of Liverpool or NIHR Alder Hey Clinical Research Facility following informed consent (information sheet Appendix A 3.3.11 and consent form Appendix A 3.3.12). Participation was approved by the University of Liverpool research ethics committee (Appendix A 2).

2.1.2 Patient clinical phenotypic data

All experiments were undertaken blinded to the patient's clinical data. Patient data were fully anonymized, but demographic and clinical phenotype data were obtained at routine clinical appointments at the time of sample collection. These data were collected using standardised data collection sheets and then uploaded onto a database based at the University of Liverpool. Data collected included: age, gender, ethnicity, current medication and disease activity assessment, informed by routine clinical blood results. The latter includes: Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), serum C3 and C4 levels, anti-dsDNA titres, the patient's Childhood Health Assessment Questionnaire (CHAQ), the BILAG global disease assessment score and specific organ system BILAG scores, and SLEDAI score.

2.2 Laboratory methods

Plasticware was purchased from Fisher Scientific or Starlab, UK. All reagents, unless otherwise stated are from Sigma Aldrich, UK.

2.2.1 Urine and serum collection

Urine samples were collected in 25 ml universals and centrifuged at 300xg for 20 min. The supernatant was aliquoted into 1.5 ml microcentrifuge tubes. Serum samples were collected in Micro tube 1.3ml Z (Sarstedt, Germany) to support blood clotting. After 30 min of clotting the sample was centrifuged at 2,000 RPM for 10 min and the serum layer was transferred into microcentrifuge tubes. All samples were stored at -80°C until use.

2.2.2 PBMC and neutrophil isolation from whole blood

Whole blood was obtained with venepuncture and collected into lithium-heparin vacutainers (Sarstedt, Germany). Samples were processed within 1.5 h of collection in the University of Liverpool Wolfson laboratories in the 'Institute in the Park', Alder Hey Children's NHS Foundation Trust. Until their use, they were left at room temperature (RT, 20-25°C) on a SLS Lab Basics Tube Roller Digital (SLS Lab Basics). Lab reagents were warmed to room temperature before use.

Five parts blood were mixed with one part HetaSep (Stemcell, UK) and were left to stand at RT for at least 30 min (**Figure 2.1 A**) until the red blood cells and plasma layer were equal (**B**). The upper layer was transferred into a fresh tube and washed with four times the volume PBS at 200xg for 10 min. The pellet was resuspended in 1 ml RPMI-1640 media with L-Glutamine (Lonza, Belgium) (referred to as RPMI-1640 from here) and layered onto 2 ml Histopaque-1077 (**C**). The leukocytes were separated at 2,000 RPM centrifugation for 20 min with a density gradient into a pellet with granulocytes and a layer of PBMCs (**D**). All media and PBMCs were carefully removed with a Pasteur pipette and the pellet was resuspended in 1 ml RPMI-1640. PBMCs for incubations with apoptotic

supernatant were washed with 7 ml PBS at 2,000 RPM for 5 min and then stored at 4°C until use (Section 2.2.4.2). Remaining red blood cells were lysed with 9 ml ammonium chloride lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate and 127 μ M EDTA). To support lysis, the tube was inverted for 1 min and left at RT for further 2 min. After a 2,000 RPM centrifugation for 5 min the supernatant was decanted, and the cell pellet was resuspended in RPMI-1640 with or without 2%/10% FCS (Life Technologies, UK). If neutrophils were further used for ultra-purification they were resuspended in 1 ml EasySep™ Buffer (Stemcell, UK) (Section 2.2.3).

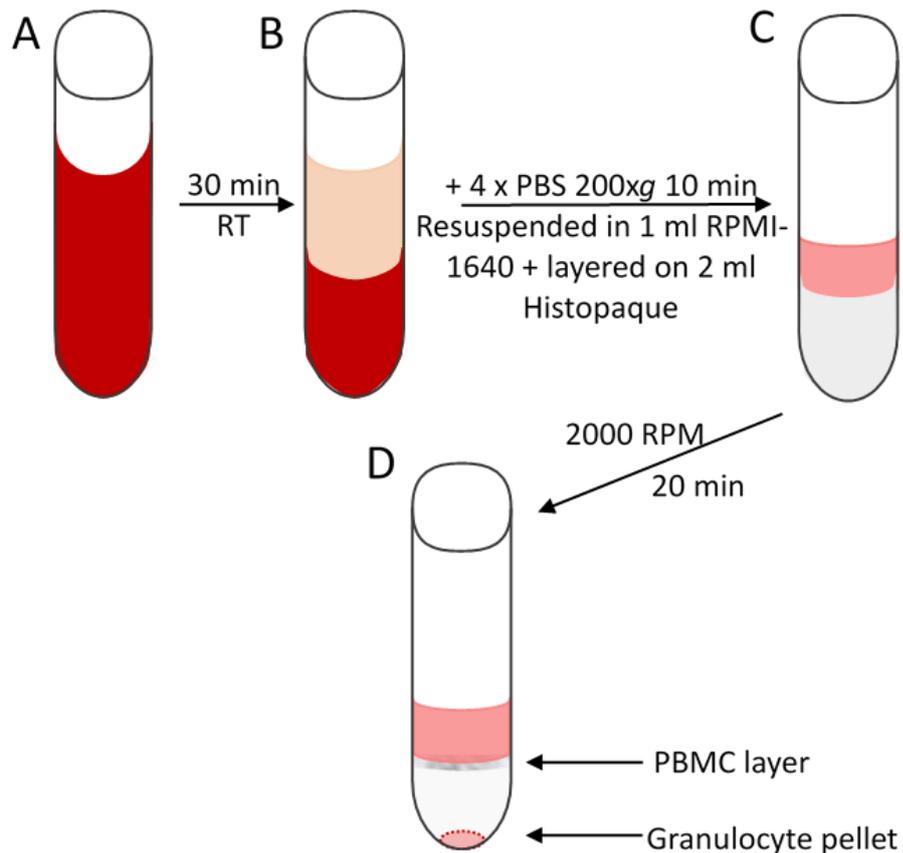


Figure 2.1: Isolation of PBMCs and granulocytes from whole blood. Five parts whole blood are mixed with one-part HetaSep and left for 30 min at RT (A). When the solution is separated equally into plasma layer and red blood cells (B), the upper layer is washed with four times the volume PBS for 10 min at 200xg. The pellet is resuspended in 1 ml RPMI-1640, layered onto 2 ml Histopaque (C) and then centrifuged 2,000 RPM for 20 min. PBMCs can be

obtained from the upper ring whereas granulocytes are pelleted on the bottom of the tube (D).

The solution of neutrophils was counted as a 1:10 dilution with a haemocytometer (Hausser Scientific, USA) and purity was assessed by flow cytometry at a 1:100 dilution. A purity of 95% neutrophils was considered sufficient.

2.2.3 Neutrophil ultra-purification by magnetic immunoselection

The EasySep™ Human Neutrophil Enrichment Kit (Stemcell, UK) was used for neutrophil purification as this is an immunomagnetic negative selection cell isolation kit. Neutrophils therefore remain undisturbed to prevent activation. All other cells are removed by binding of Tetrameric Antibody Complexes with magnetic particles (simplified in **Figure 2.2 A** as bead-labelled antibody) followed by insertion into a magnet. All bound complexes are pulled towards the magnet and the desired fraction can be decanted (**B**).

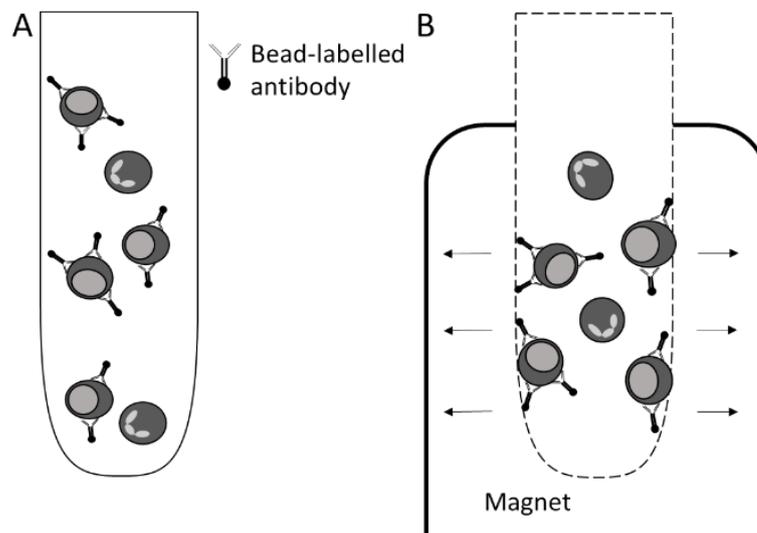


Figure 2.2: Purification of neutrophils by magnetic bead-separation using negative selection. All cell types except for neutrophils are labelled with Tetrameric Antibody complexes bound to magnetic particles which are represented as bead-labelled antibodies (A). After insertion into the magnet all labelled cells will be pulled towards the side of the tube by the magnetic force (B). Neutrophils can be poured off while all other cells remain inside the tube.

Neutrophils were adjusted to a maximum of 50×10^6 cells/ml EasySep™ Buffer, transferred to test tubes and subsequently further purified with the EasySep™ Human Neutrophil Enrichment Kit according to the manufacturer's instructions. 50 μ l EasySep™ Human Neutrophil Enrichment Cocktail was added per 1 ml solution and mixed by pipetting. After a 10 min incubation at RT, the EasySep™ Magnetic Particles were pipetted vigorously five times and 100 μ l was added per 1 ml cell solution. The suspension was mixed well and incubated for 10 min at RT. The volume was filled up to a total of 2.5 ml with EasySep™ Buffer and gently pipetted up and down. The tube was inserted into the EasySep™ Magnet and left for 5 min at RT to separate cells bound to the antibodies. The desired fraction was decanted into a fresh tube and the tube left inverted for three seconds to recover the maximum possible number of cells. This step was repeated and finally the cells were counted at a 1:10 dilution and checked for their purity on the flow cytometer at a 1:100 dilution.

2.2.4 *In vitro* stimulation of neutrophils

2.2.4.1 *Incubation with TNF α , IFN α and GM-CSF*

1×10^6 neutrophils isolated from healthy paediatric control patients were incubated in RPMI-1640 with 10% FCS with or without 1 ng/ml TNF α , 10 ng/ml IFN α (PeproTech, UK) or 5 ng/ml GM-CSF (R&D Systems, UK) and incubated at 37°C with 5% CO₂ for 30 min for TNF α otherwise 2 h for RNA expression and 1 h for TNF α otherwise 7 h for protein expression. Protein expression was measured every hour using flow cytometry as described in 5.4.3.

2.2.4.2 *Incubation with apoptotic supernatant*

1.5×10^6 neutrophils in 500 μ l RPMI-1640 were left for 24 h at 37°C with 5% CO₂ to undergo cell death. To fully assess the effect of factors released during cell death rather than effects from aged medium, “OVN medium” was used for untreated conditions. Thus, the same volume of RPMI-1640 alone was incubated for 24 h under the same conditions as the dying cells. The next day, all liquid was removed and centrifuged 200xg for 5 min. Supernatant from the neutrophils left overnight (referred to as “apoptotic supernatant”) was transferred into 1.5 ml aliquots and frozen at -80°C.

The experimental set up of incubation with apoptotic supernatant is summarized in **Figure 2.3**. In detail, neutrophils were freshly isolated from healthy adult controls as described in 2.2.2 and mixed with PBMCs at a ratio of 95:5 % or further isolated to be ultrapure (see 2.2.3). A total of 11.5×10^6 cells per condition were centrifuged at 1800 RPM for 3 min and resuspended in either OVN medium or apoptotic supernatant with 10% FCS at a concentration of 10×10^6 cells/ml. 4×10^6 cells were seeded in 24 well plates for RNA collection and 1×10^6 cells per well for the apoptosis assay and protein expression in a 96 well plate. Changes in neutrophils were then analysed as described below.

All plates were incubated at 37°C with 5% CO₂ for 2 h and 6 h. At the first time point only, RNA was collected using TRIzol (Life technologies, UK see 2.2.5.1) and cell death was measured using Annexin V/PI as described in 2.2.6.1

Apoptosis assay with Annexin V/PI. In addition, at the latter time point, cell supernatant was collected and frozen for ELISA analysis (see 2.2.9) and cell death and protein expression were measured using flow cytometry. Protein expression of CD16b, TLR2 and S100A9 was quantified with flow cytometry as described in Section 2.2.6.2.

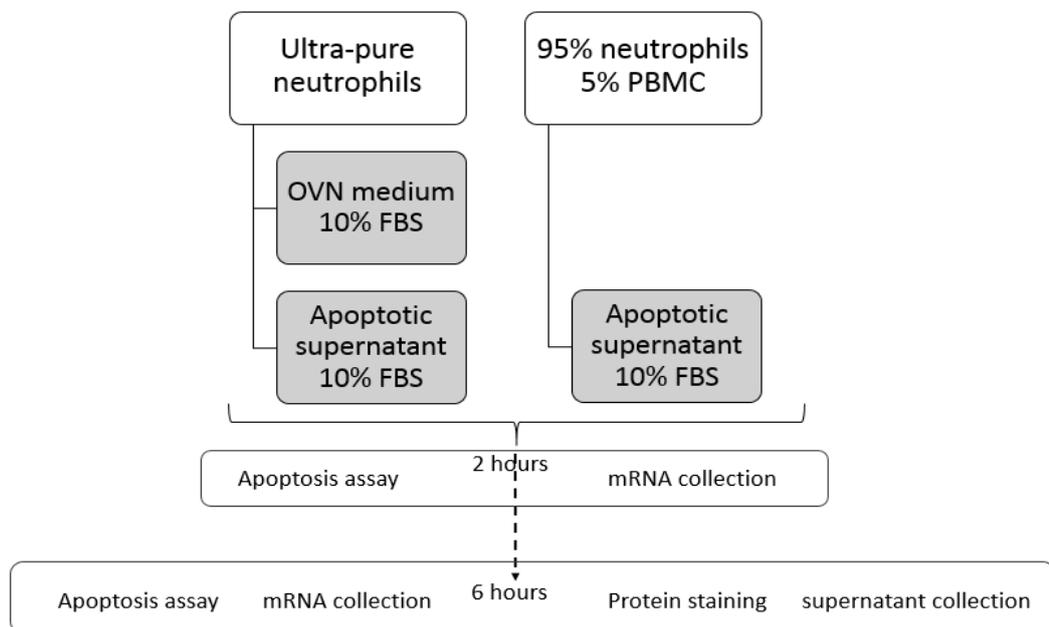


Figure 2.3: Overview of the protocol for incubation with apoptotic supernatant. Ultra-pure neutrophils were incubated with OVN medium 10% FCS or apoptotic supernatant 10% FCS. A mix of neutrophils (95%) and PBMCs (5%) was incubated with apoptotic supernatant and 10% FCS. After 2 h, apoptosis was measured and mRNA collected. At 6 h additionally supernatant was collected and neutrophils stained for TLR2, CD16b and S100A9 protein expression which was measured using flow cytometry.

2.2.5 Real-time Polymerase Chain Reaction (RT-PCR) using SYBR-Green

With real-time polymerase chain reaction (PCR) the quantity of messenger RNA (mRNA) in a sample is measured as fluorescence at the end of each cycle. There is no requirement to load the samples on a gel as would be for a standard PCR.

First RNA is transcribed into complementary DNA (cDNA) and then added to SYBR-Green dye, polymerase, nucleotides and primers specific for the gene of interest. The mixture is then placed in a thermal cycler and the amplification reaction starts with a denaturation of the cDNA and enzyme activation at 95°C as shown in **Figure 2.4**. At 60°C, primers bind to their target part of the cDNA and the polymerase elongates the targets so that a new double stranded product is made. This step is repeated 40 times and in each cycle the amount of target of interest is doubled. SYBR-Green dye intercalates into double stranded DNA (dsDNA) which results in the emission of fluorescence which can be detected by the PCR machine. The more target that is expressed in the sample, the earlier fluorescence will be detected.

After the last cycle the PCR is concluded with a melting curve to identify if the correct product has been made. The amplified product should have one specific peak dependent on its length and composition of nucleotides as seen for example in **Figure 2.5**.

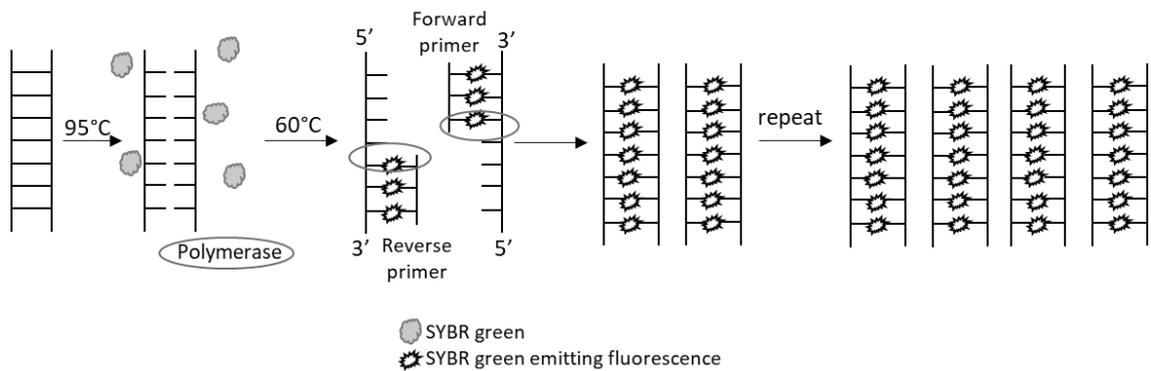


Figure 2.4: Schematic representation of real-time PCR using SYBR green. The amplification starts with a denaturation step at 95°C followed by binding of the primers and elongation at 60°C. With every repeat the amount of target doubles and SYBR-Green binds to the dsDNA which is then detected as a signal.

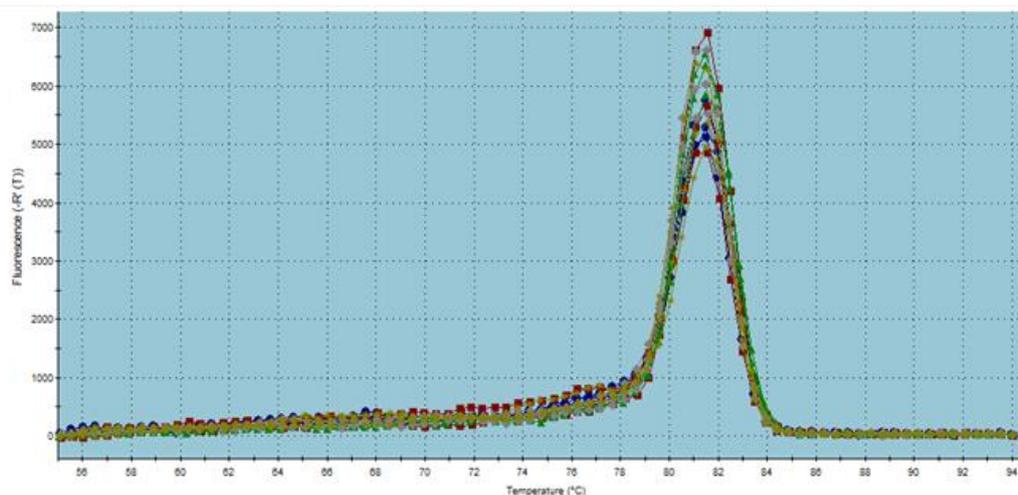


Figure 2.5: Example of a dissociation curve showing that all samples amplified the same product. PCR reactions are gradually heated up and change in fluorescence is shown on the graph for each degree Celsius. All peaks are overlapping at the same temperature, showing, that all samples contain the same product.

2.2.5.1 RNA extraction and clean-up

Cells were pelleted at 8,000xg for 5 min at RT and homogenized in TRIzol by pipetting until no cell remnants were visible. After 5 min at RT, 200 µl of chloroform was added to the tubes and they were vigorously shaken for 15 sec. This was followed by a 2-3 min incubation at RT and then the tubes were centrifuged at 12,000xg for 15 min at 4°C to separate RNA from protein and DNA. The upper clear layer containing RNA was transferred into a fresh tube and mixed with 500 µl of isopropanol. RNA was left for precipitation for 10 min at RT, approximately 1 h on ice or OVN at -20°C dependent on suitability for the experiment. RNA was pelleted by centrifugation at 12,000xg for 30 min at 4°C and the supernatant was discarded. The pellet was washed with 500 µl 70% ethanol and centrifuged at 12,000xg for 15 min at 4°C. After the supernatant was removed, the pellet was left to air dry for 1 min and then resuspended in 100 µl nuclease-free water.

The clean-up of RNA was done with the RNeasy Mini Kit (Qiagen, Germany) with an on-column DNase digest step with the RNase-free DNase set from Qiagen to minimize the risk of contamination with genomic cDNA using the manufacturer's instructions which are described below:

Before the RNA was transferred onto the membrane of the RNeasy Mini Spin Columns it was mixed with 350 µl Buffer RLT and 250 µl ethanol (96-100%) and mixed well. The spin at $\geq 10,000$ RPM for 15 sec (as used for the whole protocol unless stated otherwise), let the RNA bind to the membrane and the flow-through could be discarded. Afterwards, the column was transferred into a fresh collection tube and washed with 350 µl Buffer RW1 followed by a spin. The column was again placed in a fresh collection tube and a mixture of 10 µl DNase I stock solution and 70 µl Buffer RDD was added directly onto the column. After a 15 min incubation the membrane was washed with 350 µl Buffer RW1 and the column was placed in a fresh collection tube. Next, the membrane was washed twice with 500 µl Buffer RPE ending with a 2 min spin. The column was transferred into the final collection tube and 40 µl Nuclease-free water was put on the membrane. The tube with the column was then

centrifuged for 1 min and the flow-through placed onto the membrane again. Finally, the tube was centrifuged for 2 min and the RNA was ready for measurements of concentration and purity (A260/280 and A260/230) on the ND-1000 Spectrophotometer (Thermo Fisher Scientific).

2.2.5.2 cDNA synthesis

cDNA was synthesised either with AffinityScript qPCR cDNA Synthesis Kit from Agilent Technologies or with Precision™ Reverse Transcription Premix 2 from Primerdesign.

For the Agilent kit, 100ng RNA was topped up to a total volume of 12.5 µl with nuclease-free H₂O. For the negative control, three RNAs were randomly selected and mixed equivalent to a total of 100ng RNA and filled up with nuclease-free H₂O to 12.5 µl total volume. 3 µl random primers were added to each sample and all tubes were spun down and incubated at 65°C for 5 min. Meanwhile a mastermix was prepared with 2 µl 10x AffinityScript reverse transcriptase (RTase) buffer, 0.8 µl Deoxynucleotide Triphosphate (dNTP) mix, 0.5 µl RNase block to prevent RNase activity and 1µl AffinityScript RTase per sample. For the negative control the same mix was prepared, but without the enzyme.

The mastermix was added to the RNA-primer solution and incubated on the thermal cycler. First for 10 min at 25°C, then for 60 min at 42°C and stopped the reaction with 72°C for 15 min.

The cDNA was stored at -20°C until used.

Alternatively, the Precision™ Reverse Transcription Premix 2 from Primerdesign was used which contains an optimised blend of random nonamer primers and oligodT. The latter support mRNA transcription, while with the random nonamer primers even degraded mRNA can be transcribed.

1-10 µl RNA containing 50/100ng RNA, was mixed with 20 µl Precision™ Reverse Transcription Premix 2 and topped up with nuclease-free water to 30 µl total volume. For the negative control, three RNAs were chosen randomly and

mixed with 20 µl Reverse Transcription negative control premix which lacks the enzyme.

Tubes were spun and incubated on the thermal cycler at 42°C for 20 min and 72°C for 10 min.

Resulting cDNA was stored at -20°C.

2.2.5.3 Real-time PCR

Primers for PCR were designed and validated by Primerdesign or were chosen based on papers published in PubMed and confirmed as being appropriate for the gene of interest with Primer-BLAST tool. The published primer sequences were purchased from Eurofins (France) and Invitrogen. All primers are listed in **Table 2.1** and 100 µM stocks were diluted 1:10 for all reactions.

Target gene	Primer with forward (fwd) and reverse (rev) sequence
OAS2	fwd-CTCCTCCTTTTTCTTCCAGTCT rev-AAGCACCGAGAGCAAGATCA
IFI44L	fwd-CCTAGCCATGTGTCCTTCCA rev-GCTTTCACAGCTAGTAAGAGGACT
IFI6	fwd-CAAGGTCTAGTGACGGAGCC rev-TTTCTTACCTGCCTCCACCC
LY6E	fwd-AGGACAGGCTGCTTTGGTTT rev-AGCAGCACTGGCAAGAAGAT
DNA modifying genes: SAMSN1, SAP30, SIGLEC14, SETD7, BRCA2, TREX1, XRCC4, SRCAP	Designed and validated by Primerdesign
Housekeeping genes	Designed and validated by Primerdesign
TLR2	for-GGCCAGCAAATTACCTGTGTG rev-AGGCGGACATCCTGAACCT
S100A9	fwd-CACCCAGACACCCTGAACCA rev-GCATGATGAACTCCTCGAAGC
FCGR3B	fwd-AGTTTGAGATGCCTTGGGTTC rev-CCATCTTGGCTTGTCTGGTA
Dectin-1	fwd-AAGGATCGTGTGCTGCATCTC rev-TGGTACCCAGGACCACAGCTA
AnxA3	fwd-CCCATCAGTGGATGCTGAAG rev-TCACTAGGGCCACCATGAGA
CamK1D	fwd-GACGCCGTGTACTATCTCCA rev-GAGGACTTCAGGAGCGACAT
CR3	fwd-CCTGGTGTCTTGGTGCCC rev-TCCTTGGTGTGGCAGTACTC
b-actin (Invitrogen, USA)	fwd-AGATCAAGATCATTGCTCCTCCTG rev-CATTTGCGGTGGACGATGGA

Table 2.1: List of forward and reverse primers used for real-time PCR.

Brilliant II SYBR® Green QPCR Master Mix from Agilent Technologies and Precision®PLUS qPCR Master Mix for the Stratagene with SYBR-Green from Primerdesign were used for real-time PCR reactions. cDNA was used as a 1:10 dilution. The reaction setup is summarized in **Table 2.2**:

Agilent Technologies	Primerdesign	
5 µl 2× Brilliant II SYBR® Green QPCR Master Mix	5 µl Precision®PLUS qPCR Master Mix for the Stratagene with SYBR-Green	
0.3 µl ROX dye (1:1,000 dilution)		
0.25 µl forward primer	0.25 µl forward primer	0.5 µl primer mix if ordered from Primerdesign
0.25 µl reverse primer	0.25 µl reverse primer	
0.2 µl nuclease-free H ₂ O	2.5 µl nuclease-free H ₂ O	
Total MM: 6 µl + 4 µl cDNA template/water	Total MM: 8 µl + 2 µl cDNA template	

Table 2.2: Summary of components for real-time PCR mastermixes for both Agilent and Primerdesign kits.

Nuclease-free H₂O served as a non-template control and all samples were run in duplicate. All liquid was pulled down with a short spin. The thermal profiles run on the thermal cycler from Stratagene with the MxPro software are summarized below in **Table 2.3**:

		Agilent Technologies	Primerdesign
Denaturation/Hot start		95°C for 1 min	95°C for 2 min
Denaturation	40x	95°C for 20 sec	95°C for 10 sec
Annealing/Elongation		60°C for 20 sec	60°C for 60 sec
Dissociation curve		95°C for 1 min	
		Followed by dissociation curve starting at 55°C	

Table 2.3: Thermal profiles used for real-time PCR for Agilent and Primerdesign products. Amplification was repeated for 40 cycles and all PCRs were followed by a dissociation curve to verify that all wells contain the same product.

Resulting cycle threshold (C_T)-values were used to calculate relative expression by normalising them to at least one appropriate housekeeping gene. This method is called the Delta C_T (ΔC_T)-method. How housekeeping genes were chosen is described in the specific methods of each chapter as this was dependent on the sample type and was determined with GeNorm kit from Primerdesign.

2.2.6 Flow cytometric analysis

Cellular features such as size and granularity can be measured with a flow cytometer without fluorescence. Cells are either pulled up with a microcapillary (e.g. Guava® EasyCyte) or with a stream of liquid called sheath fluid (e.g. Beckman Coulter F500) in order to create a line of single cells as shown in **Figure 2.6**. A laser beam hits the cells at this stage and the diverted light is collected on the forward scatter (FS) and side scatter (SS).

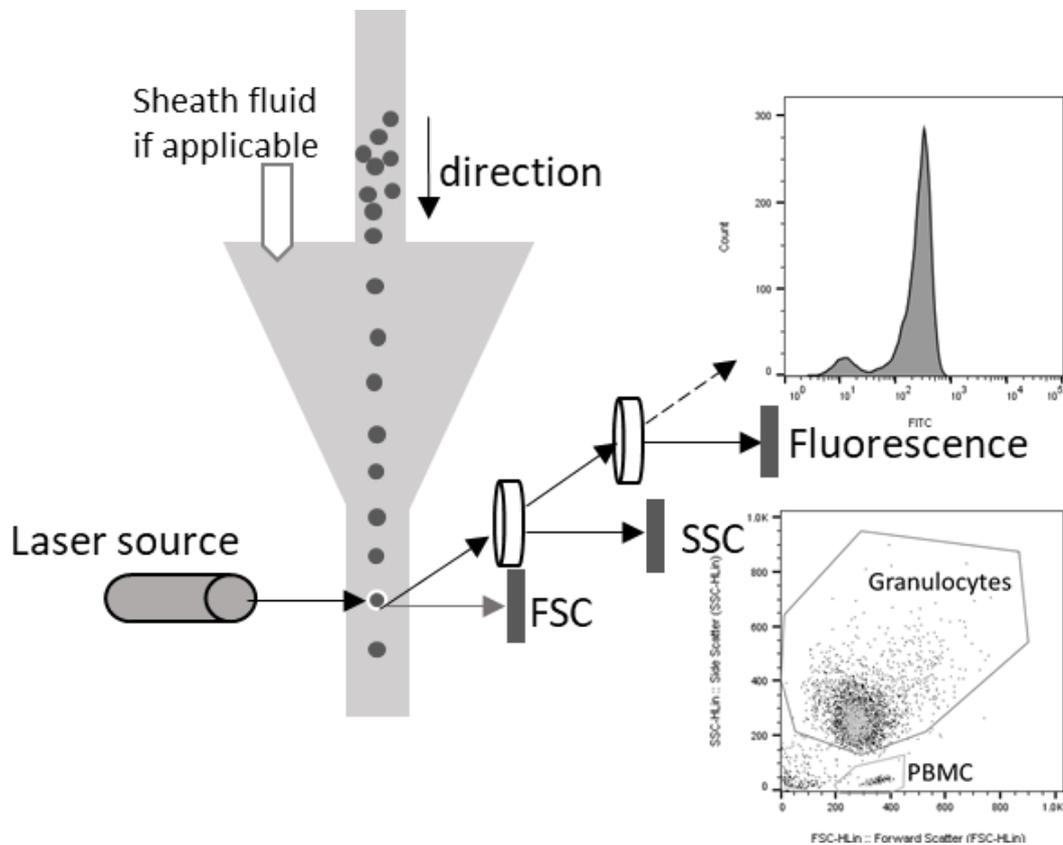


Figure 2.6: Mechanism of flow cytometry with either a microcapillary or sheath fluid. Cells are sorted into a single file and all are excited by a laser. Filters direct the emitted light dependent on the wavelength towards photomultiplier tubes which convert it into an electronic signal.

This way granular neutrophils can be distinguished from PBMCs as shown in **Figure 2.7**.

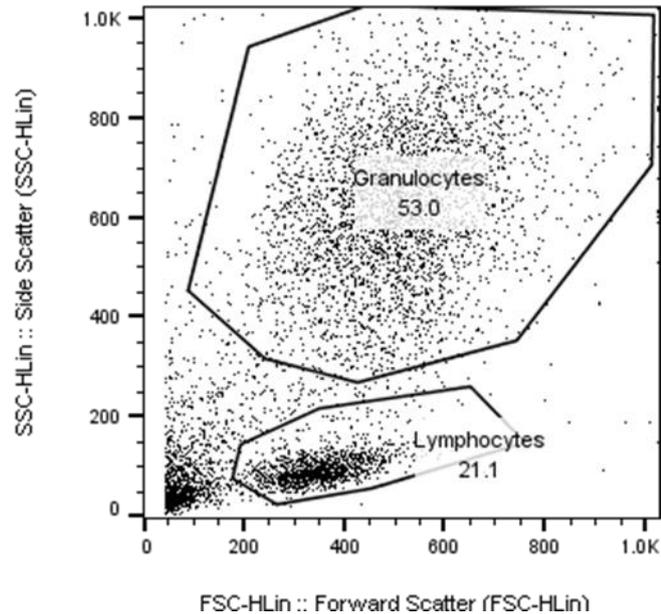


Figure 2.7: Dotplot resulting from flow cytometry measurement of forward (FSC) and sideward scatter (SSC) measurement. Cells are displayed dependent on their granularity (SSC) and their size (FSC). More granular granulocytes can be distinguished from the less granular lymphocyte/PBMC fraction.

Furthermore, cells can be stained with different fluorochromes which when excited by a laser can emit fluorescence. This signal is detected by sensors and can give information about characteristics of the cell including expression of specific proteins or their activity (e.g. uptake of pHrodo-stained bacteria). Dependent on the wavelength of the fluorochrome, different detectors collect the data which allows multiple staining to be performed at the same time.

2.2.6.1 Apoptosis assay with Annexin V/PI

Discrimination between early/late apoptosis and necrosis can be determined by staining with FITC-labelled Annexin V and propidium iodide (PI) (Sigma, USA). Upon apoptosis the membrane translocates phosphatidylserine to the outside and Annexin V can bind to it. At later stages of cell death, the membrane becomes permeable and PI can penetrate up to the point when cells are only PI positive. They would then be considered secondary necrotic.

Cells were centrifuged at 1,800 RPM for 5 min and resuspended in 200 μ l 1xAnnexin V binding buffer of which 50 μ l were then distributed into each one well of a 96-well plate. An additional 50 μ l of buffer was added followed by 1 μ l Annexin-V and incubated at 4°C in the dark for 15 min. Following the incubation, PI was added to each sample at a 1:1,000 dilution for 2 min at RT. A further 100 μ l HBSS was added and cells were analysed on the flow cytometer.

Unstained and single-stained controls were used in every run alongside the samples.

2.2.6.2 Antibody staining TLR2-PE, CD16b-FITC and S100A9-PE expression of neutrophils measured with flow cytometry

Following counting, neutrophils were centrifuged at 300xg for 5 min and resuspended to a concentration of 0.2×10^6 cells per 50 μ l in ice cold PBS/2% FCS per staining. Suspensions were left on ice for 20 min before cells for intracellular staining were fixed in 2% Paraformaldehyde for 15 min at RT. Without any further wash steps, 0.1% Triton-X 100 (BDH Limited Poole England, UK) in PBS was added with a final concentration of 0.05% which permeabilized the cells for 10 min at RT.

One part 0.1% Triton-X was added to one part permeabilized cell solution. Unfixed cells were made up to the same volume with PBS 2%FCS. Tubes were centrifuged at 300xg for 5 min and resuspended in 50 μ l ice cold PBS/2% FCS.

Antibodies were added with dilutions described in **Table 2.4**. The tubes were incubated for 30 min in the dark at 4°C. Subsequently, cells were washed twice

with 500 μ l PBS/2% with each a 300xg centrifugation for 5 min and the pellet was resuspended in 300 μ l ice cold PBS/2% FCS. All tubes were kept on ice until analysis on the flow cytometer.

Antigen	Dye	Fc-part	Dilution
TLR2	PE	IgG1, κ	1:40
CD16b	FITC	IgG2a, κ	1:10
Isotype	FITC	IgG2a, κ	1:10
Isotype (extracellular)	PE	IgG1, κ	1:80
S100A9	PE	IgG1, κ	1:20
Isotype (intracellular)	PE	IgG1, κ	1:80

Table 2.4: Antibodies used for extra- and intracellular staining for flow cytometry.

2.2.7 Phagocytosis assay

Neutrophils take up pathogens and digest them as a defence mechanism in a process called phagocytosis. pHrodo™ BioParticles® Conjugates from Life technologies (Molecular probes, UK) are pathogens or parts of pathogens and are labelled fluorescently. This can involve different phagocytic targets like gram-positive (*S.aureus*), gram-negative bacteria (*E.coli*) or even fungal particles (zymosan). Dependent on the acidity of the environment, the brightness changes which means particles are only emitting light if taken up into a phagosome where the pH is decreased. We used these particles for flow cytometric analysis and confirmed results with confocal microscopy.

1.5x10⁵ neutrophils were seeded per well in a 96 well plate in RPMI-1640 media.

The pHrodo™ BioParticles® Conjugate was resuspended in HBSS+HEPES (BDH Limited Poole England, UK) (20mM, pH 7.4) to a concentration of 1 (*S.aureus*, *E.coli*) and 0.5 (zymosan) mg/ml. The cells were incubated with 0.3 µg *S.aureus*, *E.coli* or 0.5 µg zymosan per 1x10⁵ cells and with or without 10%

JSLE/paediatric control serum. After a 5 min or 20 min incubation at 37°C, 5%CO₂ (or on ice as a negative control), the plates were centrifuged at 1800 RPM for 3 min and resuspended in HBSS + HEPES. They were kept on ice until analysis.

2.2.8 Assessment of phagocytosis using confocal microscopy

Visualization for phagocytosis experiments was achieved with incubations on coverslips and analysis under the confocal microscope (Leica TCS SPE with the optical microscope Leica DM 2500) as detailed below.

2×10^5 neutrophils were plated into 24 well plates on autoclaved cover slips and left for approximately 45 min to settle and adhere to the glass. Afterwards the cells were treated as described above in Section 2.2.7, but incubated for 20 min, having established that 5 min was insufficient time for all particles to settle to get comparable results between flow cytometry and confocal microscopy assays. After the 20 min incubation all medium was removed and 200 μ l 1xTBS diluted from a 10x solution (100 mM Tris-Base (Fisher Scientific, UK), 1.5 M NaCl (Fisher Scientific, UK) (pH 7.5)) was added. Coverslips were removed from the liquid with forceps and mounted on a glass slide with ProLong™ Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Fisher Scientific, USA). The slides were left to set for at least 1 h and then examined under the confocal microscope.

Using the technique of confocal microscopy as shown in **Figure 2.8**, fluorescence from antibody-bound fluorophores or from DAPI, a DNA staining dye, can be detected. The fluorochromes are excited with lasers, but instead of using a wide field as in conventional microscopy, samples are scanned with light beams. Out-of-focus light is prevented with pinhole apertures and only fluorescence of the sample that is in focus will reach the photomultiplier which generates a signal with low background noise.

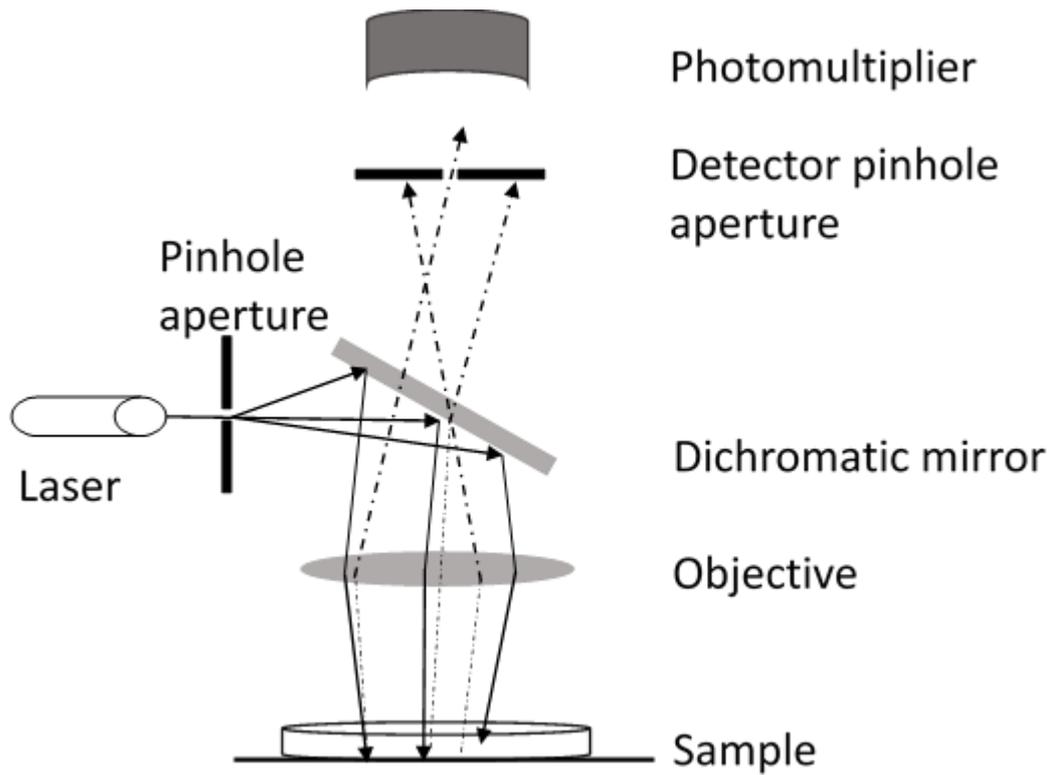


Figure 2.8: Principles of confocal microscopy. Light from a laser is directed through a pinhole aperture onto a mirror and reaches the sample through the objective. Fluorescence is emitted from fluorochrome labelled antibodies bound to proteins on the sample. Out-of-focus light is stopped by the detector pinhole aperture and only in-focus light will reach the photomultiplier.

2.2.9 Sandwich ELISA

For a sandwich enzyme-linked immunosorbent assay (ELISA) (see **Figure 2.9**), primary antibodies against the specific antigen are first immobilized onto a polystyrene plate. The plate is then blocked against non-specific binding with bovine serum albumin (BSA). Serum, plasma or supernatants are then incubated on the plates alongside a serial dilution of standards. The antigen of interest present in these samples will bind to the antibodies on the plates. Afterwards, the secondary, biotinylated antibody is added, which is directed specifically against the antigen. The enzyme horseradish peroxidase (HRP) is coupled to avidin which binds to the biotinylated antibody. This ensures that an HRP substrate such as 3,3', 5,5' tetramethylbenzidine (TMB) turns blue if the specific coupled antibodies are present. This process needs to be stopped with H_2SO_4 which also turns the blue colour into yellow and the plate can be read at a wavelength of 450 nm. With the optical density values from the serial dilutions using a four-parameter logistic (4-PL) curve-fit, a standard curve is generated making it possible to calculate all sample concentrations. All results were analysed on <http://www.elisaanalysis.com/>.

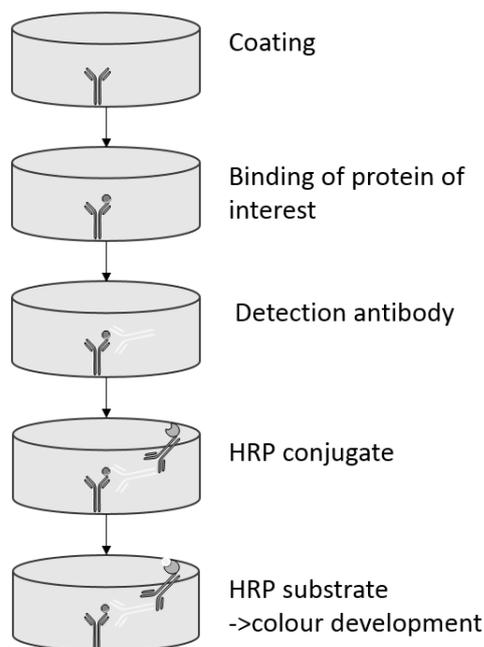


Figure 2.9: Procedure to perform a sandwich ELISA. At first wells are coated with capture antibodies, then the protein of interest and standards are bound followed by the detection antibody. The HRP conjugate binds to the second antibody and will ensure colour development when coming into contact with the HRP substrate.

2.2.9.1 ELISA detecting CD16b

The CD16b in serum or released into supernatant was measured with the Aviva systems biology FCGR3B ELISA kit and all reagents were brought to RT before use. Serum was diluted 1:160, all other samples 1:2 and standards were serially dilute. 100 μ l diluted samples were plated onto the precoated ELISA plates. An incubation of 12 h at RT was followed by incubation with 100 μ l of 1X Biotinylated FCGR3B Detector Antibody for 1 h at 37°C and three subsequent washes. For all washes for CD16b ELISA, wash buffer was used which was supplied with the kit. 100 μ l of 1X Avidin-HRP Conjugate were added into each well and incubated at 37°C for 1 h. The plates were washed five times and the reaction was started with 90 μ l of TMB substrate at RT and stopped after 15 min with 50 μ l Stop solution.

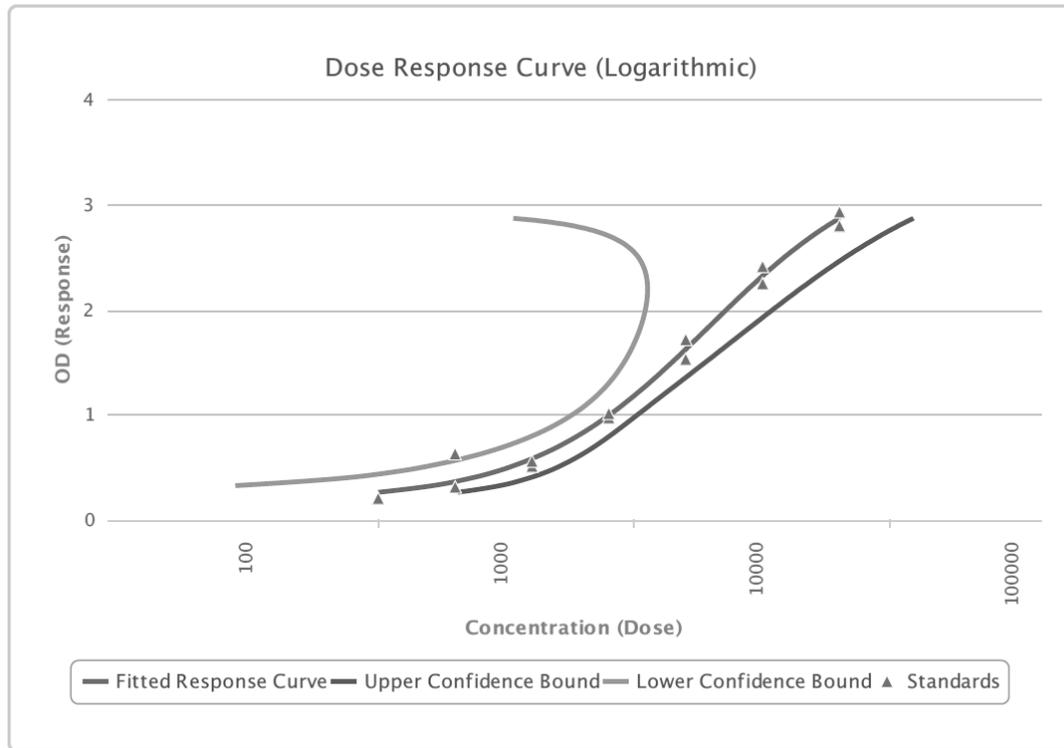


Figure 2.10: Example for a standard curve resulting from CD16b standards displayed as 4-Parameter Logistic Regression. Standards were plated in duplicate and the R^2 was calculated to be 0.99. The resulting curve was used to determine concentrations of samples.

2.2.9.2 ELISA detecting $TNF\alpha$, S100A9 and S100A8/S100A9

ELISA kits for $TNF\alpha$, S100A9 and the S100A8/S100A9 complex were all purchased from R&D systems, UK and used as described. 100 μ l of 1x Capture Antibody diluted in filtered PBS was plated on a 96 well plate and incubated at RT OVN. Plates were washed three times and then blocked for 1 h at RT with reagent diluent (1% BSA in PBS). The plate was washed three times and the 100 μ l of sample, either undiluted or diluted in reagent diluent, or serially diluted standards were plated out and incubated OVN at RT or for 2 h at 37°C. Subsequently, plates were washed three times and incubated with 100 μ l working concentration of Detection antibody diluted in reagent diluent. Plates were washed three times before 100 μ l of the working dilution of Streptavidin-

HRP were added to each well. Subsequently, plates were incubated 20 min in the dark at RT and washed again three times. Finally, 100 μ l of Substrate Solution (one-part H₂O₂:one-part TMB BD Biosciences, UK) were placed into each well and the reaction was stopped after 12-15 min with 50 μ l of Stop Solution (2N H₂SO₄, BDH Limited Poole England, UK).

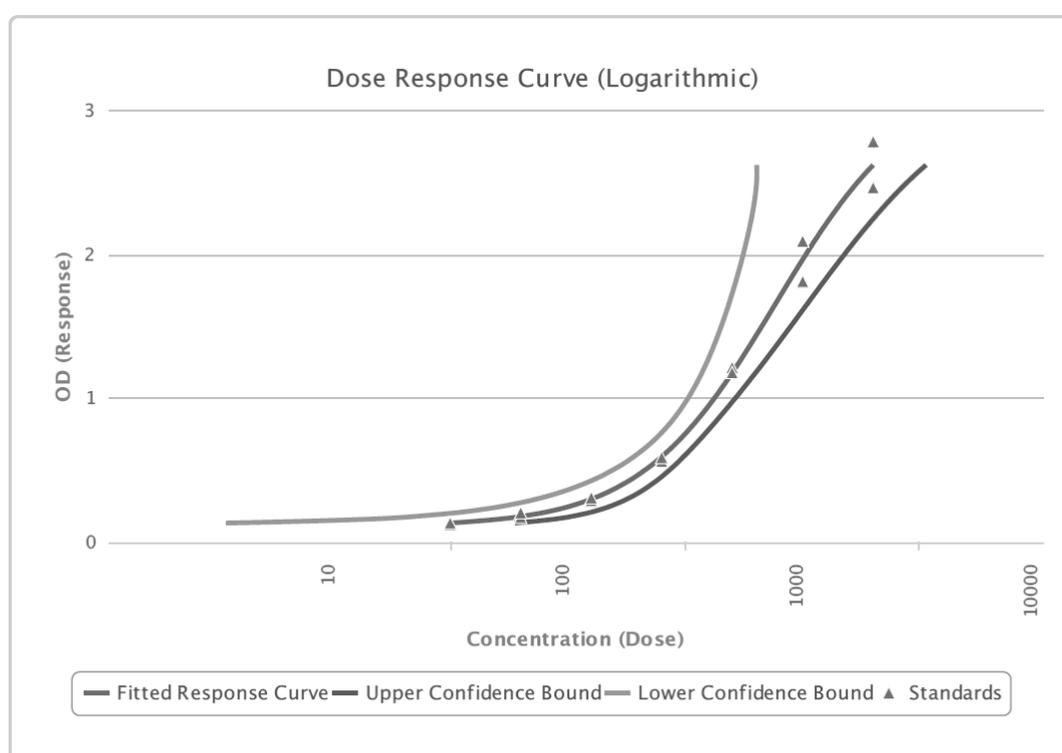


Figure 2.11: Standard curve resulting from S100A9 standards displayed as 4-Parameter Logistic Regression. Standards were plated in duplicate and the R² was calculated to be 0.992. The resulting curve was used to determine concentrations of samples.

2.2.10 Whole blood assays

2.2.10.1 Nucleosome purification

Nucleosomes were isolated from purchased chicken blood in Alsever's solution (TCS Biosciences Ltd Botolph Claydon, UK) as avian erythrocytes still contain nucleosomes in contrast to mammalian erythrocytes. Two falcon tubes each with 50 ml of chicken blood were centrifuged for 10 min at 1800xg and after the supernatant was discarded the pellet was washed twice in buffer A (0.08 M NaCl/0.02 M EDTA, pH 7.5). Subsequently the erythrocytes were resuspended in 23 ml buffer A and lysed by adding 23 ml of 1.5% Triton X-100 (in buffer A diluted). Tubes were left on ice until the lysates were carefully layered onto sucrose gradients consisting of 2 ml 2.25M sucrose and 15 ml 1.7M sucrose in ultracentrifuge tubes. This was followed by ultracentrifugation at 113,000xg for 90 min at 4°C. The sucrose solutions were removed carefully, and residues were removed with a Kimwipe using a metal spatula. Nucleosomes were carefully resuspended in 20 ml buffer A. Afterwards, nucleosomes were centrifuged for 15 min at 2,000xg at 4°C and supernatants removed. Nucleosomes were then washed twice with 15 ml of 50 mM Tris-Cl (pH 7.9) at 2,000xg for 15 min at 4°C. After all the supernatant was removed, nucleosomes were resuspended in 15 ml cold distilled water and sonicated at 4°C until reaching a homogenous solution.

The concentration of DNA was measured using the ND-1000 Spectrophotometer and adjusted to 1 µg/µl with 0.1 mM EDTA. Nucleosomes were stored at -80°C until used.

2.2.10.2 Whole blood stimulation

1 ml of blood was taken by venepuncture and incubated with 1 µl Brefeldin A (1000x stock solution) with or without 20 µg nucleosomes and with or without anti-IFNα receptor (IFNAR)-antibody or isotype control for 5 h at 37°C with 5% CO₂. Afterwards cells were stained for TLR2, CD16b and S100A9 (see 2.2.6.2). For RNA extraction the incubations without Brefeldin were used and processed as described in 2.2.10.3.

Blood was blocked with cold PBS/ 2% BSA in a 1:1 ratio in test tubes for 30 min on ice. Then 1 ml of 1x BD FACS™ Lysing Solution (BD Biosciences, UK) was added and the solution was thoroughly pipetted up and down to support red blood cell lysis continued by a 10 min incubation at RT. Tubes were centrifuged 500xg for 3 min. All subsequent centrifugations were performed with these conditions. Subsequently, the pellet was washed with 1 ml PBS 2% BSA and cells were either stained extracellularly for TLR2, CD16b or isotype control (see 2.2.6.2) or permeabilised with a concentration of 0.05% Triton-X 100 in PBS for 10 min. Cells were spun down in a final concentration of 0.075% Triton-X and the supernatant was poured off. Subsequently, cells were stained for intracellular CD16b or S100A9 as described in 2.2.6.2 or cells were resuspended only in the remaining liquid and 5 µl IFN α -FITC antibody or IgG κ 1 isotype control (Miltenyi, UK). After 1 h incubation at RT cells were washed twice with each 1 ml PBS/2% BSA. For analysis using the flow cytometer cells were resuspended in 250 µl PBS/2% BSA.

2.2.10.3 Whole blood RNA extraction

Due to the very small volumes used for whole blood assays, a separation into neutrophils and PBMCs was not advisable prior to RNA extraction. Therefore, the blood not needed for antibody staining was mixed one-part HetaSep, five parts blood. After 30 min at RT the leukocyte-rich layer was transferred into a fresh tube and washed with PBS at 200xg for 10 min. The resulting pellet was resuspended in 1 ml RPMI-1640 and 9 ml ammonium chloride lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate and 127µM EDTA) was added. The tube was inverted for 1 min to support red blood cell lysis followed by a 2 min incubation at RT. This was followed by a centrifugation at 2,000 RPM for 5 min and cells were further processed for RNA extraction as described in 2.2.5.1.

2.3 Statistical Analyses

Statistical analysis was performed with GraphPad Prism 6.

Comparison between two non-parametric groups which were not-matched was analysed with the Mann-Whitney test. If the same conditions were given for more than two groups, the Kruskal-Wallis test followed by Dunn's *post hoc* test was applied. For two matched groups that were non-parametric, a Kruskal-Wallis matched-pairs signed rank test was performed and for multiple groups the Friedman test was used with Dunn's multiple comparisons *post hoc* test. P-values of <0.05 were considered significant in these tests.

2.4 Metabolomics using ^1H NMR spectroscopy

^1H NMR is, as further described in Chapter 3, a technique often used for a non-targeted screening for differences in metabolites. In this study it was used as a metabolic profiling tool to distinguish between different autoimmune diseases and further compare if JSLE IFN high and JSLE IFN low patients are similar enough to be classified as one disease or if they should be considered as separate diseases.

If atomic mass and atomic number are both even the nucleus cannot be detected with NMR as only spinning nuclei can be observed. The ^1H isotope is the most naturally abundant isotope (99.98%) and therefore often used for NMR spectroscopy. Nuclei spin and create a magnetic field with their electrical charge. When an external magnetic field is applied these spins are aligned to the field. This requires energy which is released once the magnetic field is turned off again and which can be detected. Dependent on the chemical environment of the proton, a frequency shift can be observed as electrons would shield the proton from the magnetic field. In real terms this means that ^1H bonded to different chemicals have different resonance frequencies. These frequencies are measured in Mz (longitudinal magnetisation; z-direction), and converted into parts per million (ppm) and moving of positions along the ppm-axis are called chemical shifts. Electronegative groups, e.g. methyl, result in a low chemical shift and electropositive groups, e.g. aldehydes or aromatics, in a high chemical shift.

As neighbouring nuclei can affect with their orientation the magnetic field force experienced by the proton, a so-called spin-spin coupling effect can be observed which causes a split in the signal causing multiple peaks known as doublets, triplets, etc.

Different pulse sequences of the magnet can be applied to the nuclei and one of them is the 1D Carr-Purcell-Meiboom-Gill sequence (1D CPMG) which records resonances of low molecular weight molecules (<5000Da) whilst removing (attenuating) resonances from macromolecules such as lipoproteins. This sequence is very useful for biofluids such as serum which contains multiple

albumins and was also applied in our study. An example can be seen in **Figure 2.12**.

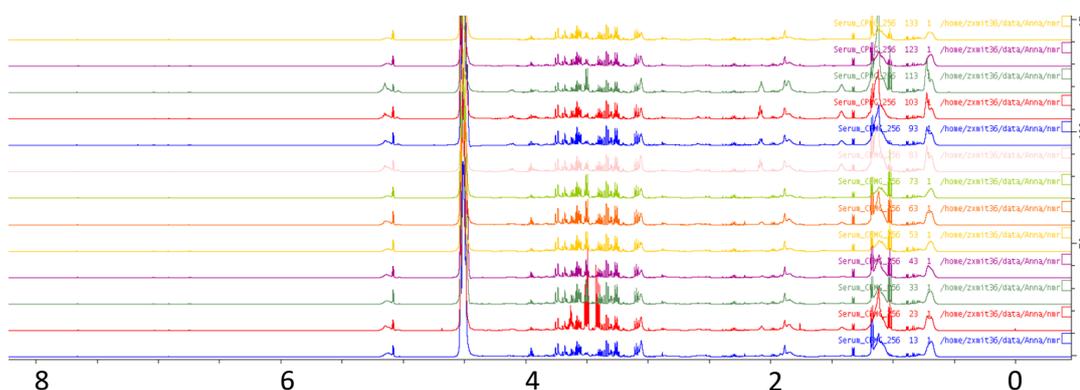


Figure 2.12: Examples of CPMG spectra obtained with ^1H NMR. Range shown here covers 0 ppm on the right to 8 ppm on the left and each line represents a different sample.

2.4.1 Serum

Blood is a body fluid rich in immune cells and should therefore be rich in metabolites resulting from these cells. For investigations comparing JIA, JSLE and healthy paediatric control patients, serum samples were compared regarding their ^1H NMR spectra. Samples were obtained and stored as described in Section 2.2.1.

2.4.1.1 Sample preparation

Healthy paediatric control, JIA and JSLE serum samples were thawed on ice and then mixed 1:1 with a phosphate buffer containing 19.75% 1 M Sodium

Phosphate pH 7.4 (19 parts $\text{NaH}_2\text{PO}_4^{3-}$ and 81 parts $\text{Na}_2\text{HPO}_4^{3-}$), 79.75% distilled H_2O and 0.5% 1.2 M NaN_3 . All samples were vortexed for 1 min and centrifuged 13,000xg for 2 min at 13°C. Trimethylsilylpropanoic acid (TSP) cannot be added as a reference standard to serum as it is a protein-rich solution. It would result in binding to protein and consequently in an attenuated signal that will also be shifted according to the amount of albumin present.

2.4.1.2 Spectral acquisition, processing and referencing

Sealed NMR tubes containing serum samples were run on the Bruker Avance III 600 MHz equipped with CryoProbe at 37°C. Temperature was calibrated using the methanol thermometer [208]. Samples were locked onto $^2\text{H}_2\text{O}$ and 1D CPMG NMR spectra were acquired at 600.13MHz with 4s interscan delay. Serum were acquired with 32 and 256 scans, 4 dummy scans and a spectral width of 20 ppm (3.067s acquisition time per scan) and in 3 mm outer diameter tubes.

All acquired spectra were processed using auto-routines in Topspin-3.1 (Bruker, UK), time domain data was converted with Fourier transformation and the resulting resonance spectra were automatically phased and baseline corrected.

Serum samples were then referenced to the glucose peak (left peak of the doublet at 5.244 ppm) to ensure precise alignment of peaks (alignment shown in **Figure 2.13**). This is a robust method frequently used for serum samples [209].

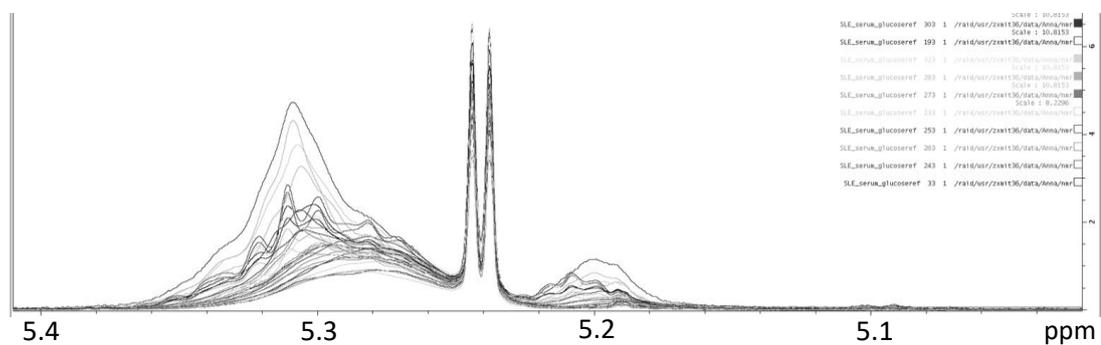


Figure 2.13: All spectra for serum are referenced to glucose. All samples were overlaid and show that they are all referenced to glucose with the left lobe at 5.24 ppm.

2.4.1.3 Creating a pattern file using TopSpin® and Chenomx Profiler® (version 8.2 standard)

Peaks within the spectra were assigned to 'bins', also known as buckets defined by left and right boundaries (example shown in **Figure 2.14**). As many peaks as possible were annotated with Chenomx Profiler® software and peak boundaries in ppm combined with annotations employed as pattern files (Appendix C 1.1). The Amix® software integrated the area under the peaks within the variable sized buckets and scaled the pattern region to region size and the peaks to the total intensity of the spectrum. These peak intensities are now summarized in a bucket table. All unidentified peaks were considered unknown and the annotated patterns were then integrated into the bucket table. These bucket tables were used for analysis with the computing software R.

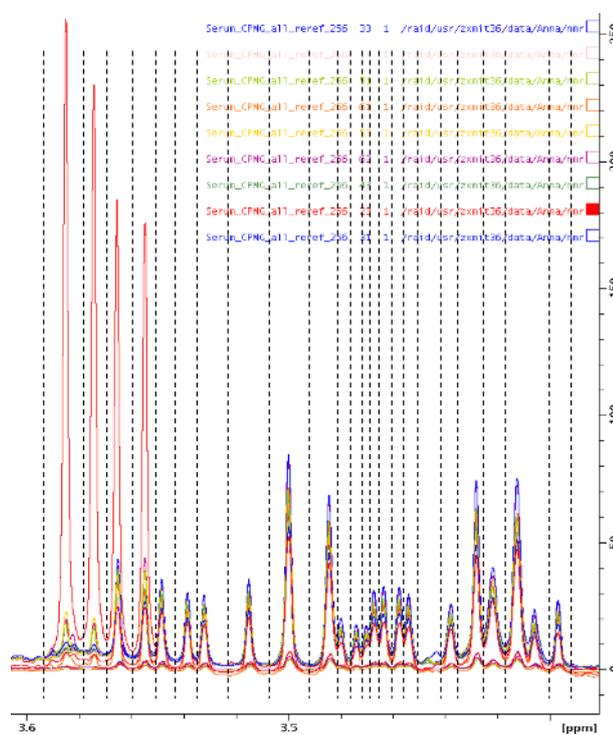


Figure 2.14: Selection of boundaries for all spectra showing 3.4 ppm to 3.6 ppm as an example. Each bucket is indicated by a dashed line.

Metabolites can create several peaks in a spectrum, depending on their chemical structure. Depending on the number of peaks for each metabolite and if these peaks were singlets or doublets etc., a metabolite could be annotated with more or less confidence. This is summarized in a confidence table in Appendix C 1.2. Furthermore, spectra annotated in Chenomx Profiler® were confirmed where possible by in-house library and level of identification recorded in the confidence table as recommended by the metabolomics society initiative [210].

2.4.2 Urine

Urine is a waste product of the body and should therefore be representative of a wide range of bodily processes and metabolic pathways. For investigations urine samples from JIA, JSLE and healthy paediatric control patients were compared regarding their ¹H NMR spectra. Samples were obtained and prepared as described in 2.2.1.

2.4.2.1 Sample preparation

Urine samples from healthy paediatric were thawed on ice before they were centrifuged 6,000xg for 5 min. Compensation for variation of pH was achieved by 50% dilution of samples with urine buffer (1M phosphate, pH 7.4, 20% ²H₂O, 2.4mM NaN₃, 200uM TSP) resulting in urine samples with final concentration of 500mM phosphate, pH 7.4, 10% ²H₂O, 1.2mM NaN₃, 100μM TSP. This results in precipitation of some metabolites but was still considered as suitable as peaks would be subjected to even stronger pH shifts otherwise. Urine is generally low in protein and can be spiked with TSP which can be used in downstream analysis as a reference peak. Samples were vortexed for 1 min, then centrifuged 13,000xg for 2 min at RT and lastly pipetted into NMR tubes with 5 mm outer diameter. Until spectroscopy was performed tubes were left at 4°C.

2.4.2.2 Spectral acquisition and referencing

Urine samples in sealed NMR tubes were run on the Bruker Avance III 600 MHz equipped with CryoProbe at 27°C, calibrated with a methanol thermometer. Samples were locked onto $^2\text{H}_2\text{O}$ and 1D CPMG NMR spectra were acquired at 600.13MHz with 4s interscan delay. Urine were acquired with 32 and 256 scans, 4 dummy scans and a spectral width 30 ppm (1.818s acquisition time per scan) in 5 mm outer diameter tubes. Processing of spectra was undertaken with Topspin-3.1 (Bruker, UK) using Fourier transformation, they were automatically phased and baseline corrected. All samples were referenced to the TSP peak at 0 ppm (**Figure 2.15**).

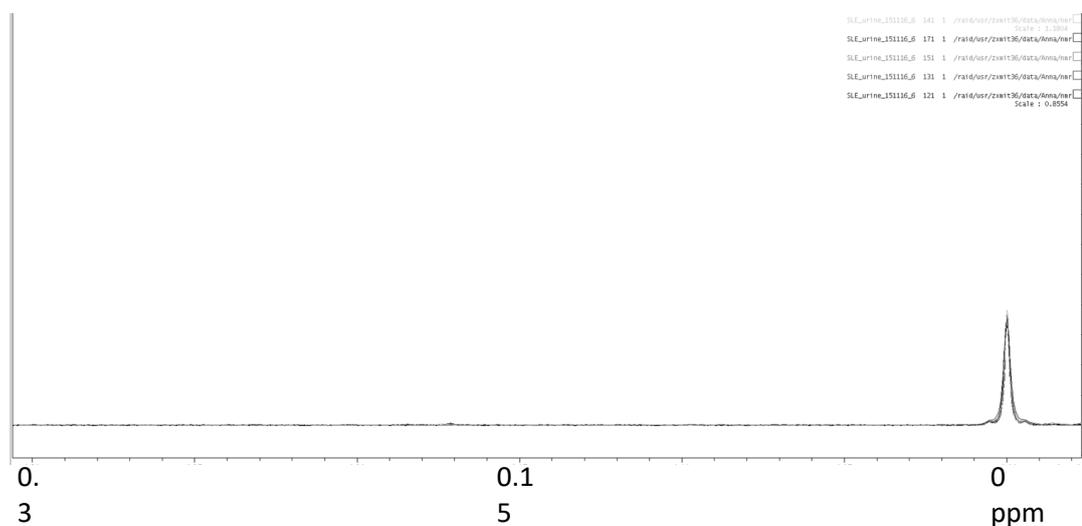


Figure 2.15: All spectra for urine are referenced to TSP. Samples were overlaid and show that they are all referenced to TSP at 0 ppm.

2.4.2.3 Creating a pattern file using Chenomx Profiler® (version 8.2 standard)

Despite buffering the urine samples, there was a wide variation in the sample pH and ionic strength which resulted in shifts in the peaks (**Figure 2.16**). Chenomx Profiler® software not only annotates peaks, but also deconvolutes the peaks (see **Figure 2.17**) and calculates the area of each metabolite that is annotated. As many metabolites as possible were annotated in every spectrum and for each sample the area for the metabolites was exported into Excel. These results were collated into one sheet and further used in the same way as the values obtained from bucket tables for serum and then analysed in R.

As mentioned in Section 2.4.1.3, metabolites can create several peaks in a spectrum, depending on their chemical structure. Depending on the number of peaks for each metabolite and if these peaks were singlets or doublets etc. a metabolite was annotated with more or less confidence. Again, where possible metabolites were matched to those present in the in-house library and assigned an identification level as recommended by the metabolomics society initiative [210]. This is summarized in a confidence table in Appendix C 1.3.

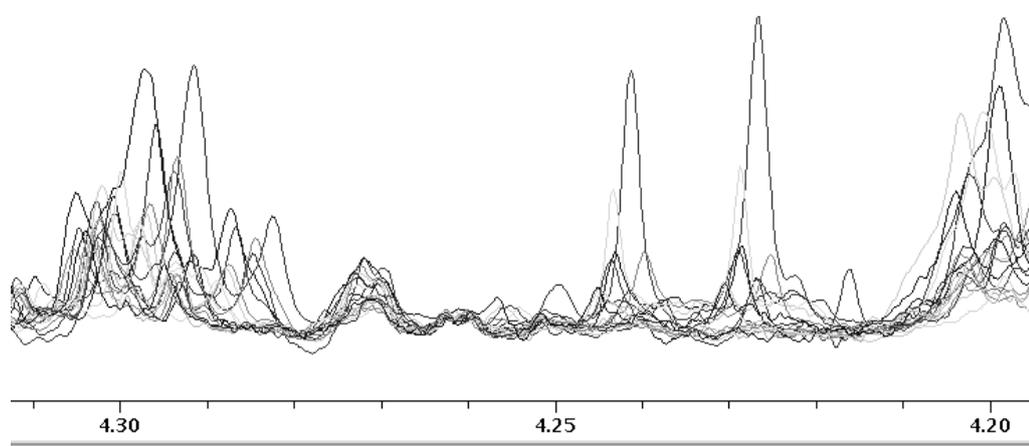


Figure 2.16: Urine spectra showing strong variation between the samples due to pH differences and ionic strength. Each line represents a different spectrum obtained from urine samples from different patients. Most peaks of the same metabolites are not overlapping.

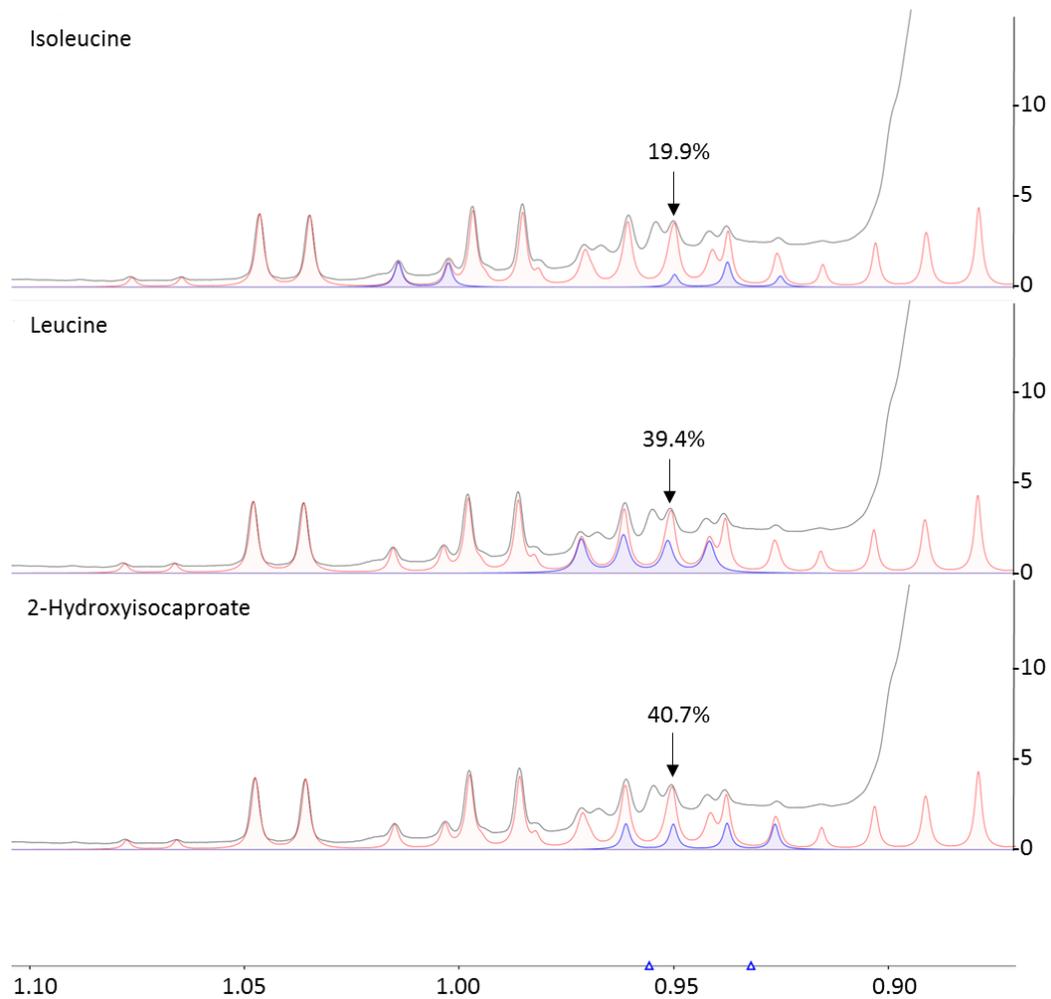


Figure 2.17: Example for deconvolution of a peak. The black line indicates the acquired spectrum. The red area represents the sum of all annotated metabolites and the blue area shows the selected metabolite. This peak here consists for example of 19.9% Isoleucine, 39.4% Leucine and 40.7% 2-Hydroxyisocaproate. The total area for each metabolite can be extracted and used as a method for quantification.

2.4.3 Analysis using R

For all analysis unidentified peaks were excluded to avoid false positive discovery solely due to metabolites potentially present in patient medication. The peak identified as “Ethanol” as described in “3 Metabolite profiles of serum and urine in autoimmune disease” was excluded. Statistical analysis as well as graphical representation of Principal Component Analysis (PCA) and partial least squares discriminant analysis (PLS-DA) was performed with statistical packages within R (Team R, Cranfield, UK) and scripts were kindly provided by the Computational Biology facility technology directorate from the University of Liverpool (UK).

2.4.3.1 Normalization and scaling

Statistical analysis could only be undertaken if spectra were normalized and scaled as this makes them comparable. While serum samples were normalized using probabilistic quotient normalization (PQN), the urine samples were normalized to their creatinine peak as applied in many papers (e.g. [211], [212]). Both serum and urine samples were pareto-scaled, meaning division by the square root of the standard deviation to prevent bias towards larger variables.

2.4.3.2 PCA and PLS-DA

PCA reduces the dimensions of variables with an algorithm and presents them as principal components (PC) in two dimensions to simplify analysis. Thereby it allows to compare similarities between samples visually and we assessed outliers with this type of analysis. Each PC has a value stating how much of the variation within the data set is explained by it (**Figure 2.18A**).

PCA is considered a non-supervised approach as it clusters samples excluding knowledge about their categories/groups. In contrast, PLS-DA takes the different groups into account and tests with multiple linear regression techniques if it is possible to discriminate between the sample types by the

given variables. This effectively maximises the variance between groups while minimising the variance within groups. This is also visualized on a two-dimensional map. Values between 0 and 1 give indications about robustness of the model (R^2) and between -1 and 1 giving indications about predictive power (Q^2) **(B)**. Variable Importance in Projection (VIP) scores are also produced ranking the buckets/peaks which impacted the separation most and were therefore considered most influential for differences between the diseases **(C)**.

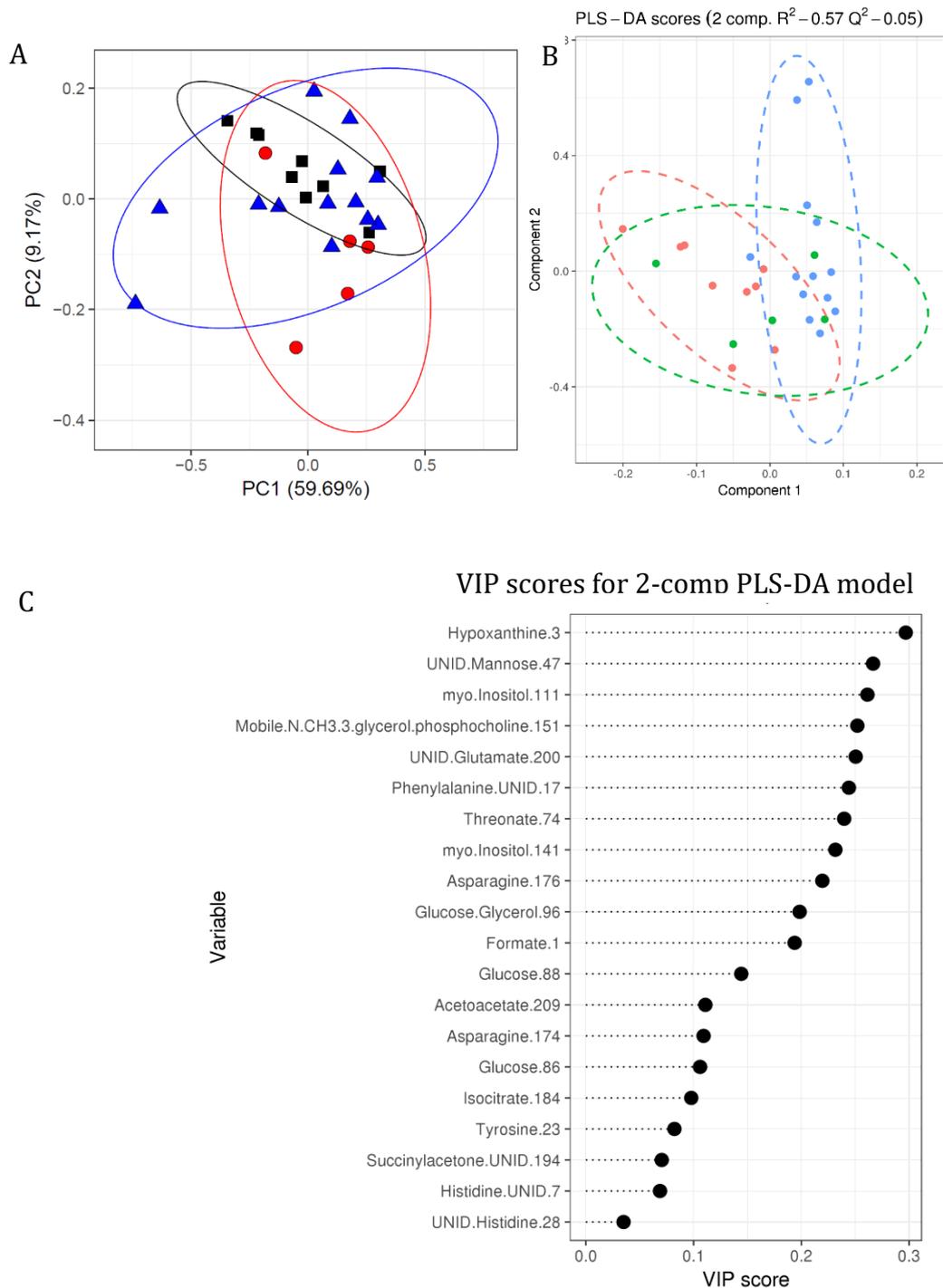


Figure 2.18: Examples for PCA, PLS-DA and a VIP score. PCA shows samples distributed on the plot depending on principal component (PC) 1 and PC2 (A). Together both principal components can explain 68.9% of the samples. The same samples were then analysed for their ability to be separated with PLS-DA (B). The black squares, red circles and blue triangles in PCA equal the red, green and blue circles respectively in PLS-DA. Two components give the best results for a PLS-DA with a R^2 of 0.57 and a Q^2 of 0.05. Dependent on their impact on the PLS-DA metabolites are listed in the VIP score with the most influential on the top.

2.4.3.3 Univariate statistical analysis of metabolomics data

Statistical significance was assessed between three groups using ANOVA. The appropriate *post hoc* test would be Tukey of Fisher, a multiple test adjustment was also applied using Benjamini-Hochberg false discovery rate adjustment. This method adjusts for multiple testing which occurs as there are many metabolites identified, but also each metabolite has at least one peak. As many metabolites have several peaks this adjustment can be very conservative and due to our small sample size, we might lose information. P-values were therefore used unadjusted with a value less than 0.05 as important.

2.5 Summary

In this chapter it was presented how this study used samples arising from the UK JSLE Cohort Study and Repository from patients and healthy controls to seek to improve stratification of JSLE patients and understand the role of PGS and IGS in these patients. How patients were recruited, what clinical data was collected and how samples were processed was presented.

Furthermore, laboratory and metabolomic methods were explained, background of these methods described, and the general approach to analyses undertaken of results has been reported.

3 Metabolite profiles of serum and urine in autoimmune diseases

3.1 Introduction

So far, “-Omics” approaches to study pathogenesis of autoimmune diseases such as JSLE and JIA focus more on genomics and transcriptomics rather than metabolomics.

3.1.1 Metabolomics

There are different ways to investigate diseases, but each method is limited in the data that it creates and its interpretation (see Section 1.5). For example, transcriptional approaches (as described in Section 1.5.2 and Chapter 4 - IFN-induced and phagocytosis-related gene signature in JSLE neutrophils) measure mRNA levels which give information about which proteins a cell is about to make and therefore what a cell is trying to do. Investigating protein expression of the cells and tissues is often necessary and common to confirm that transcriptional changes have an impact [4]. Activity assays are used for enzymes or functional assays to enable the action of cells to be investigated. This can make the influence of the studied genes clearer, but this is only an artificial condition with inhibitors or stimulants defined by the experimental conditions. Furthermore, most tests only look at a subset of proteins or cells and cannot evaluate the complete biological system with all its interactions. The environment created in the assay with cytokines added nutrients etc. and chosen cell types could be considered a biased system.

The study of all metabolites present in a type of cell, tissue or body fluid is called metabolomics (see Section 1.5.4). Metabolomics (in both serum and urine) facilitates the measurement of side and end products of biochemical pathways to provide detail of the *in vivo* metabolic state. Metabolites may be the results of an immediate response to a stimulus or event, but can also indicate accumulation of metabolites from ongoing processes.

3.1.2 ^1H NMR spectroscopy of serum and urine

Metabolomics methods can use either nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) – see Section 1.5.4. The latter is a good tool for a targeted project such as the identification of biomarkers. It is used to quantify a class of molecules such as fatty acids or amino acids. Even though MS is a very sensitive technique, the required preselection of the targets leads to a loss of information. An initial separation with gas or liquid chromatography is usually applied before the ionization step necessary for mass-spectrometry. This causes increased preparation time which can cause a loss of metabolites due to instability of molecules [213].

NMR spectroscopy on the other hand is an untargeted approach, but metabolites can only be detected if they are present in high abundance and this technique is therefore less sensitive than MS. Low abundance metabolites may be lost in the background noise. Nevertheless, because there is no separation step prior to the run on the spectrometer, generally a high abundance of molecules is reached. There is further preselection due to the choice of atom. The nucleus of atoms is made up of neutrons and protons called nucleons of which each has $\frac{1}{2}$ spin. Only atoms with an odd number of nucleons can be detected with NMR as only these possess $\frac{1}{2}$ spins which are affected by a magnetic field. ^1H NMR is the most popular choice because of the richness of ^1H compared to ^{13}C or ^{14}N . Other advantages of ^1H NMR spectroscopy are reproducibility, cost-effectiveness, and it being an unbiased and non-destructive method [84].

Serum and urine are the body fluids that are relatively easy to obtain with urine collection being particularly non-invasive. Systemic fluids can offer information on entire biological systems rather than skewing results to a particular cell or tissue type, which may only show part of the biological response. Additionally they give insight and have been used to investigate drug treatments for a long time with ^1H NMR ([214],[215]). Protocols have been more standardized over time and are now used to explore many different research questions [216].

3.1.3 JIA and JSLE

Even though both JIA and JSLE are autoimmune diseases, generally more prominent in females, that share symptoms including fatigue, fever or joint pain and autoantibodies, they also differ significantly and are two distinct diseases. At the same time, both reflect a spectrum of disease manifestations that can differ widely between individuals affected. JSLE is considered the archetypal multisystem autoimmune disease which can affect any organ system including the kidney, skin or brain for example (see Section 1.3). JIA comprises a group of disorders in which clinical manifestations are generally characterised by joint inflammation (such as oligoarticular and polyarticular forms of JIA, or enthesitis-related arthritis) joint involvement, but there is also a form in which systemic features are typically present (see Section 1.2).

While investigations of disease mechanisms in JIA and JSLE using metabolomics are very limited (see Section 1.5.5), there have been several studies focusing on adult-onset arthritis or exploring metabolic changes due to treatments ([217], [218]). There have been a number of studies in adult-onset SLE patients where this approach has been used, for example using mass spectrometry or focusing on lupus nephritis ([87], [85]). Nevertheless, metabolomic investigation of a paediatric cohort is very limited for both JIA and JSLE and usually either focused on serum or urine metabolites.

This chapter will focus on the metabolomics investigation of JSLE. In addition, JIA will be compared and contrasted as an alternative autoimmune disease characterised by tissue inflammation, and both these compared to healthy paediatric control patients, based on their metabolite profile both in urine and serum. JSLE patients will be further divided into those expressing an IFN high and an IFN low gene signature. By investigating metabolites that differ in expression to control patients (either increased or decreased) the aim is to detect potential dysregulated pathways that may provide further insight into inflammatory diseases such as JIA and particularly the autoimmune disease JSLE.

From past studies, it is known that neutrophils from JSLE and JIA patients behave differently compared to paediatric healthy control patients regarding their cell death or presence of LDGs ([137], [19]). Changes in metabolites may be detected in the serum or urine of patients with JIA or JSLE as neutrophils are the most abundant cell type in blood.

3.2 Chapter hypothesis

The hypothesis for this chapter therefore is that JSLE and JIA patients can be distinguished from non-diseased individuals (healthy controls) by their metabolite profiles in urine and serum.

Studies have previously indicated that patients can be distinguished, based on their IFN gene expression profile, which will be termed here IFN 'high' and IFN 'low', dependent on their relative expression of their IFN-induced gene signature [56]. It is hypothesised in this study, that IFN low patients are more similar to control patients than IFN high patients and that a separation based on the IFN signature is possible. As metabolites are representative of processes occurring in the body, it is hypothesised that these analyses may provide additional evidence regarding dysregulated pathways in JSLE and JIA patients.

3.3 Objectives

The objectives for this chapter were:

- **Objective 1:** To determine if serum or urine is better for building a model to distinguish between metabolite profiles of JIA, JSLE and healthy paediatric control patients, and between the JSLE IFN high and low subtypes and healthy paediatric control patients.
- **Objective 2:** To identify potential pathways differentially regulated between disease groups and healthy paediatric control patients, as well as between the IFN subtypes of JSLE patients.

3.4 Chapter specific methods

3.4.1 Experimental plan

Serum and urine samples were obtained from children (disease onset before 16th birthday) with JIA (n=5 for serum, n=4 for urine), with JSLE (n=13, n=8) and from paediatric healthy controls (n=9, n=4) – see Section 2.1. JSLE patients were subsequently separated into IFN low (n=4 for serum, n=3 for urine) and IFN high (n=6 for serum, n=4 for urine) dependent on their interferon (IFN) signature as described elsewhere [57], [219] [220]. The IFN signature for patients studied was obtained from transcriptomics analysis conducted by our group previously. The samples were run at 37°C for serum and at 27°C for urine on a Bruker 600 MHz AvanceIII spectrometer equipped with CryoProbe. Resulting ¹H NMR spectra were analysed with Topspin, Chenomx NMR Suite and R.

Resulting PC and PLS-D analyses (see Section 2.4.3.2) can help to establish disease models to predict disease groups and differences between metabolites can help to identify underlying mechanisms that may influence the development of the disease. Comparison of models based on serum or urine metabolites can identify which body fluid is more useful in the study of these diseases.

3.4.2 Analysis of serum

Acquisition and processing of the samples was performed as stated in “2.2.3 Metabolomics using ¹H NMR spectroscopy”. Serum was then referenced to glucose, a metabolite intrinsically present in all serum samples, normalized using probabilistic quotient normalization and afterwards pareto-scaled as described in “2.2.3.1.2 Spectral acquisition and referencing” and “2.2.3.3.1 Normalization and scaling”. When analysing serum metabolites in JSLE, it is important to exclude patients who have been treated with Rituximab. B-cell depletion therapy has a major impact on metabolites in serum (Surace and Midgley *et al.*, in preparation). To observe differences, it is therefore important to only use sera from patients who had not received Rituximab treatment in the last 12 months.

Table 3.1 lists the patients included in the comparison between JSLE, JIA and controls. The same patients of the JSLE and control group were used previously for transcriptomics analysis from our group and were able to be separated into interferon-induced gene signature high “IFN high” and low “IFN low” patients.

Study ID	SLEDAI	Age	Gender	Ethnicity	Treatment
C314a	-	13	Female	WB	None
C316a	-	9	Female	WB	None
C322a	-	14	Female	WB	None
C341a	-	14	Male	WB	None
C348a	-	15	Female	WB	None
C367a	-	13	Male	WB	None
C368a	-	17	Male	WB	None
C372a	-	13	Female	WB	None
C374a	-	15	Female	WB	None
J15d (O)	-	12	Female	WB	Corticosteroid - joint injection
J66b (P)	-	9	Female	WB	Biologics
J84a (P)	-	9	Female	WB	Corticosteroid - joint injection
J85a (O)	-	9	Female	Other Asian background	DMARD
J89a (P)	-	9	Male	WB	No medication
L30l	10	10	Male	Bangladeshi	HCQ 200, MMF 1500, Pred 5, Infliximab IV, Risedronale 35, Azithromycin 250, Omeprazole 20, Calcichew D3, fluconazole 250, diclofenac 50
<i>L43j</i>	4	13	Female	WB	HCQ 200, MMF 2400, Pred 5, Omeprazole 20, folic acid 5
<i>L62i</i>	16	14	Female	WB	HCQ 200 and MMF 1500
L64i	7	17	Female	WB	Pred 10
L65f	8	17	Female	WB	HCQ 400 MMF 1000
L66i	4	16	Female	WB	HCQ 200 MMF 2000
L67a	4	9	Female	African	MMF 500
L68e	16	15	Female	WB	HCQ 400, MMF 2000, Pred 10
<i>L69a</i>	12	17	Male	Other	HCQ 200, MMF 1500, Pred 15 Cyclophosphamide IV
L73a	4	14	Female	No info	HCQ 400 MMF 2000

Table 3.1: List of patients included for serum analysis. Group (C=healthy control, J=JIA, L=JSLE), disease activity (SLEDAI) age, gender, ethnicity and medication is stated for patients. Brackets indicate subtype of JIA (O=Oligoarthritis, P=Polyarthritis, S=systemic arthritis). The most frequent medication were: Hydroxychloroquine (HCQ), Mycophenolate (MMF) and Prednisolone (Pred). Doses written after medication are in mg. The font of JSLE patients indicates IFN high (bold) and IFN low (italic) status.

Initial analysis revealed a strong difference between JSLE patients and the other groups due to peaks annotated in Chenomx Profiler® as “Ethanol”. Also some other studies found ethanol in their results [85] potentially as a contamination. As we found this mainly in JSLE patients, this was investigated further.

A major difference between JSLE patients and JIA patients and paediatric controls, besides the disease itself, is the medication most patients receive. Most commonly given to JSLE patients is hydroxychloroquine, a DMARD administered to downregulate the immune system. This was run on the 700 MHz AVANCE™III HD spectrometer. When analysing the obtained spectrum in Chenomx Profiler® it also recognized the “Ethanol” peaks as seen in **Figure 3.1**. Black lines indicate the acquired spectrum opened in Chenomx Profiler®. The blue line shows the triplet of ethanol around 1.2 ppm where it was also found in serum of JSLE patients.

In order to avoid a model primarily due to separation by treatment, the peaks from ethanol (or ethanol-like molecules) have been excluded from further analysis. Additionally, analysis was only performed with annotated metabolites as we assumed unknown peaks could also be artefacts of medication and skew the analysis.

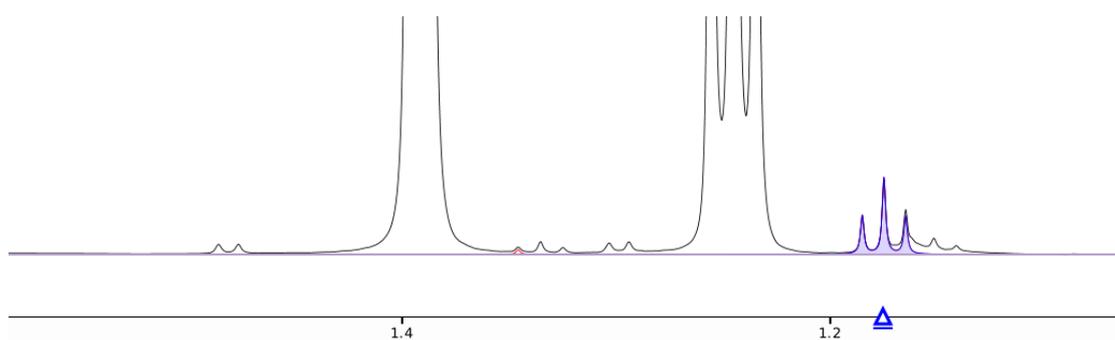


Figure 3.1: "Ethanol" peak detected in the Hydroxychloroquine spectrum. Hydroxychloroquine was run on the 700 MHz AVANCE™III HD and the resulting spectrum is indicated by black lines. One of the metabolites detected within the hydroxychloroquine spectrum by Chenomx Profiler® was Ethanol as it was annotated in serum of JSLE patients. The triplet shown here at 1.2 ppm in blue is part of the predicted ethanol peaks.

3.4.3 Analysis of urine

Urine is considered to be a waste product of the body and urinary metabolites can be indicators for processes that occur in excess. In investigating disease processes in children, and as a source of determining potential biomarkers of disease, it is a potentially ideal medium, as it is far easier to obtain than taking additional blood tests from patients. Urine has a lower content of protein compared to serum, so adding TSP as an internal reference peak is therefore possible. The single peak of TSP was set as 0 ppm and all other peaks were referenced to it. Variation in pH and ionic strength (salt content) between the samples can affect peak positions as measured for example by Platzer *et al* (2014). For assignment and analysis, it was therefore necessary to annotate metabolites in Chenomx Profiler® to overcome these problems. This process needs extreme care to match entire metabolite profile in a process of trial and error leading to deconvolution of peaks to give one relative abundance per metabolite as opposed to the multiple values per metabolite possible in homeostatic serum sample. The exported area under the peaks was used to measure relative abundance of metabolite a as described in 2.1.2. As no patient had acute kidney disease it was decided that creatinine is a valid peak to normalize for so that dilution factors could be taken into account [221].

Patients included in the study of urine are listed in **Table 3.2**. The three groups include healthy paediatric controls, JIA patients and JSLE patients. The classification of IFN high and IFN low JSLE patients is shown (as mentioned in Section 3.4.2) and further discussed in Chapter 4.

Study ID	SLEDAI	Age	Gender	Ethnicity	Treatment
C316a	-	9	Female	WB	None
C367a	-	13	Male	WB	None
C368a	-	17	Male	WB	None
C372a	-	13	Female	WB	None
J9b (O)		20	Male	WB	Corticosteroids, biologics, NSAIDs and DMARDS
J66b (P)		9	Female	WB	Biologics
J90a (S)		9	Male	WB	Corticosteroid - oral
J105a (P)		13	Female	WB	Biologics
L34h (not tested on IFN)	22	7	Male	WB	HCQ 400 and warfarin 6
<i>L62h</i>	0	12	Female	WB	HCQ 200, MMF 1500, Pred 2,5
L64i	7	17	Female	WB	Pred 10
L65f	8	17	Female	WB	HCQ 400, MMF 1000
L66g	5	6	Female	WB	HCQ 200, MMF 2000, Pred 5, statin
L68e	16	15	Female	WB	HCQ 400, MMF 2000, Pred 10
<i>L69a</i>	12	17	Male	Other	HCQ 200, MMF 1500, Pred 15 Cyclophosphamide - no info on dose
L73a	4	14	Female	No info	HCQ 400, MMF 2000
<i>L43s</i>	16	16	Female	WB	HCQ 200, MMF 2000, Pred 5

Table 3.2: List of patients included for urine analysis. For each patient group (C=healthy control, J=JIA, L=JSLE), disease activity (SLEDAI) age, gender, ethnicity and medication is stated. Brackets behind JIA patients give information about the type of JIA (O=Oligoarthritis, P=Polyarthritis, S=systemic arthritis). The most frequent medication were: Hydroxychloroquine (HCQ), Mycophenolate (MMF) and Prednisolone (Pred). Doses written after medication are in mg. The font of JSLE patients is dependent on their classification as IFN high (bold) and IFN low (italic).

3.5 Results

3.5.1 Serum metabolomics

3.5.1.1 Differences between the serum metabolome of JSLE patients, JIA patients and of healthy paediatric controls

In this study, serum from JIA patients with mainly non-systemic onset subtypes was used to observe any differences between a systemic disease with neutrophil involvement (JSLE) and one with mainly joint involvement (JIA).

Principal component analysis (PCA) looks at the entire variance within the spectra and clusters the patients according to the metabolites explaining most of the differences by grouping the variances into principal components (PC). Due to the differences between gender, age and ethnicity within the groups as well as between the groups, a high variance between the metabolites and no immediate clustering looking at the unsupervised PCA which is indiscriminate to patient diagnoses was expected. This type of analysis consequently was used to find outliers to prevent false positive detection by skewed data (see **Figure 3.2**). Applying PCA to the data showed that for 63.5% of the variance (PC1 51.6% + PC2 11.9%) there were no outliers detected. All samples were within the 95% confidence interval and as expected there was no clustering of the groups observed.

The aim was to differentiate between the diseases and healthy controls based on metabolite profiles. Similarly, it would be possible to separate patients regarding their age, body-mass-index or other characteristics. Nonetheless, for this thesis the main question was if metabolites can cluster patients into their disease groups from a group of heterogeneous patients. Therefore, a PLS-DA was applied to investigate if a separation by the serum metabolome was possible (see **Figure 3.3**). The separation of JSLE, JIA and healthy paediatric control patients required three components. It was deemed very robust with an R^2 of 0.82 (**Figure 3.3 (A)**). The R^2 value ranges from 0-1 and a value of >0.5 is considered a strong model. At the same time, the predictive power is very low with a Q^2 of only 0.18. The Q^2 value can be between -1 to 1 and a value > 0.4 is considered acceptable for biological systems [222]. In the Variable Importance

in Projection (VIP) **(B)** score Arginine/N-Acetylserotonin.233 and Succinylacetone/UNID.194 are the two highest ranked metabolites. Both are overlapped peaks and hence, it cannot be distinguished which, or if possibly both metabolites are responsible for the differentiation between the study groups. Arginine, Asparagine and Succinylacetone are the main contributors to this separation as they have additional peaks in the spectrum which appear in the VIP score further down.

The 10 most influential metabolites are listed in **Table 3.3** also showing importance of Glucose and Hypoxanthine. These metabolites were increased in JSLE patients compared to controls but decreased or unchanged for JIA patients compared to controls.

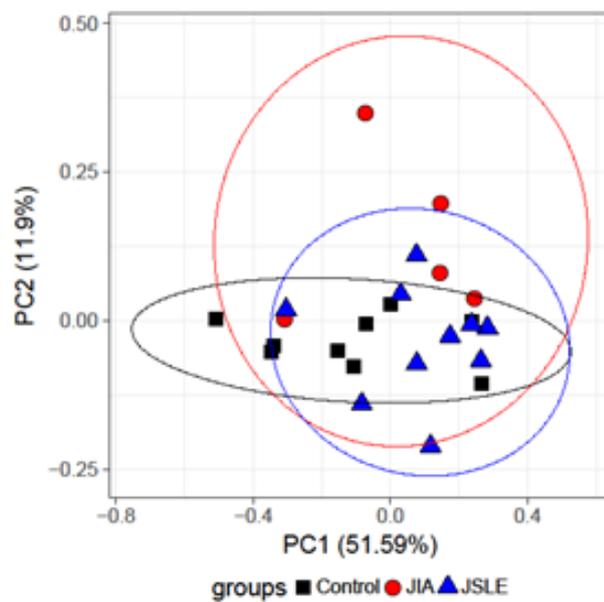


Figure 3.2: PCA of serum spectra of JSLE, JIA and healthy paediatric control patients. No outliers were observed for either JSLE (blue triangles, n=10), JIA (red circles, n=5) or healthy controls (black squares, n=9) as for each group the patients were within their 95% confidence interval indicated by a blue, red or black circle respectively. 63.5% of the overall variance could be explained with PC1 and PC2.

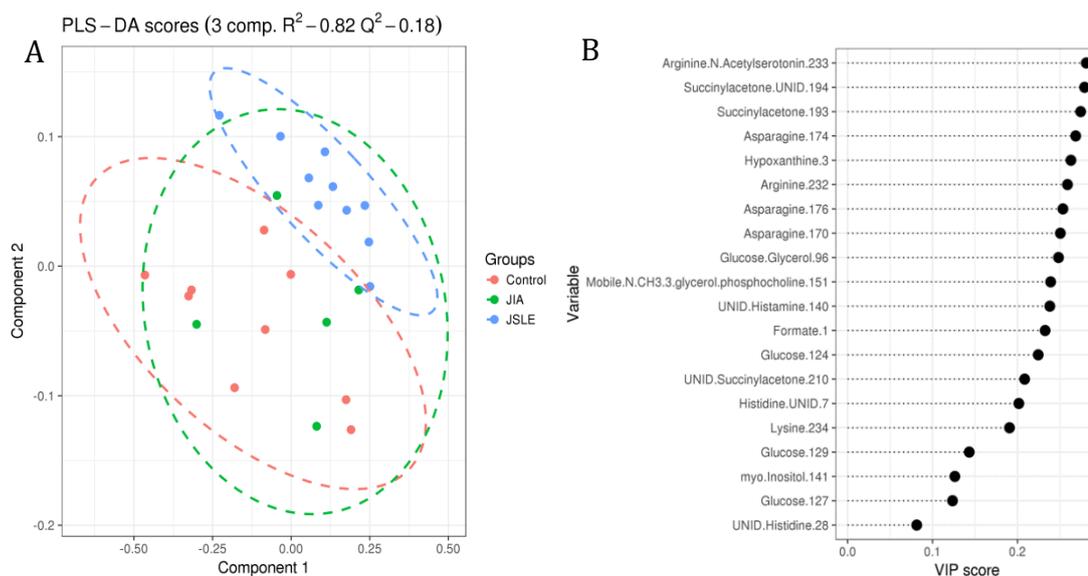


Figure 3.3: PLS-DA of serum metabolome of JSLE, JIA and healthy control patients. Patients (JSLE $n=10$, JIA $n=5$, Control $n=9$) were separated with a supervised analysis (A) by the differences of their metabolites. Three components give the best separation with highest robustness ($R^2=0.82$) and highest predictive power ($Q^2=0.18$). Metabolites responsible for separation are shown in the VIP score for the first component (B). Metabolites are annotated by name and their unique identifying number to allow for multiple NMR peaks per molecule.

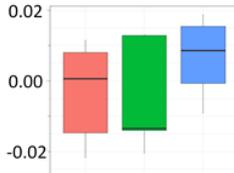
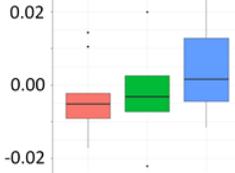
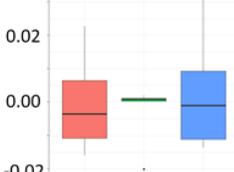
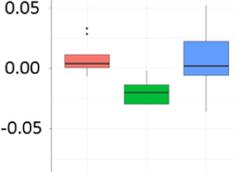
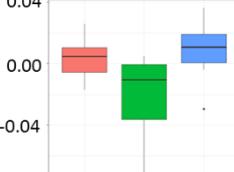
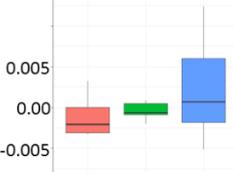
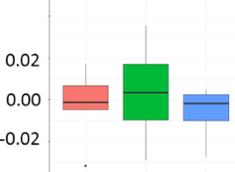
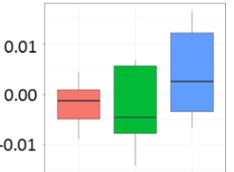
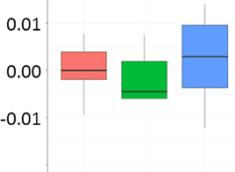
Metabolite and numbers of peaks within the TOP 20 VIP score with representative boxplot of control (red), JIA (green) and JSLE (blue) serum			
Arginine 2		Asparagine 3	
Formate 1		Glucose 4	
Glycerol 1 (overlapped)		Histidine/UNID 2	
Hypoxanthine 1		Mobile lipids/ phosphocholine 1 (overlapped)	
Succinylacetone 3		UNID/Histamine 1	

Table 3.3: The ten most influential metabolites for PLS-DA of metabolites in JIA, JSLE and control serum. The metabolites are listed in alphabetical order with one representative boxplot, but each metabolite may have several peaks within the VIP score. The number of peaks is stated after the metabolites. The boxplots show abundance on the Y-axis for the metabolite in serum of healthy paediatric control patients (red), JIA (green) and JSLE patients (blue).

Metabolites described in **Table 3.3** are central to the separation between the three disease groups. Yet, there are more significant differences noted between the three groups as shown by one-way ANOVA between the three groups (see **Table 3.4**). Some of the metabolites are overlapping with the VIP score like Glucose, a main separator in PLS-DA was also found significant with ANOVA.

Annotated peak	p-value	adjusted p-value	Significant for Tukey's <i>post hoc</i> analysis between
Acetoacetate.209	0.00033	0.02785	JIA-Ctrl, JSLE-JIA
Alanine.244	0.00852	0.11871	JIA-Ctrl, JSLE-JIA
Creatine_phosphate.79	0.04883	0.23243	JIA - Ctrl (p=0.1), JSLE-Ctrl (p=0.08)
Glucose.124	0.02813	0.15415	JIA-Ctrl, JSLE-JIA
Glucose.127	0.00468	0.10618	JIA-Ctrl, JSLE-JIA
Glucose.129	0.01509	0.15415	JIA-Ctrl, JSLE-JIA
Glucose.98	0.03122	0.16152	JIA-Ctrl, JSLE-JIA
Glucose.Glycerol.96	0.02765	0.15415	JSLE-JIA
Glucose.UNID.128	0.00535	0.10618	JIA-Ctrl, JSLE-JIA
Glutamine.UNID.219	0.01321	0.15415	JSLE-Ctrl
Histidine.Arabinose.72	0.0285	0.15415	JSLE-Ctrl
Leucine.Isoleucine.UNID.271	0.02648	0.15415	JSLE-Ctrl
Lysine.163	0.02803	0.15415	JSLE-JIA
Mannose.81	0.00898	0.11871	JIA-Ctrl, JSLE-Ctrl
N-Acetylserotonin.15	0.00459	0.10618	JSLE-Ctrl, JSLE-JIA
N-Acetylserotonin. Phenylalanine.14	0.0007	0.02785	JSLE-Ctrl, JSLE-JIA
Ornithine.158	0.01802	0.15415	JSLE-Ctrl
Ornithine.160	0.02468	0.15415	JSLE-Ctrl
Phenylalanine.12	0.02277	0.15415	JSLE-JIA
Phenylalanine.UNID.17	0.0216	0.15415	JSLE-JIA
Succinylacetone.UNID.194	0.00048	0.02785	JIA-Ctrl, JSLE-JIA
Trimethylamine_N.oxide. Glucose.146	0.02755	0.15415	JIA-Ctrl, JSLE-JIA
UNID.Glutamine.188	0.00646	0.10982	JSLE-Ctrl
UNID.Lysine.164	0.02848	0.15415	JSLE-JIA
UNID.Valine.212	0.04871	0.23243	JSLE-Ctrl

Table 3.4: Significant peaks of metabolites from serum of JSLE, JIA and healthy paediatric control patients. Metabolites were tested with one-way ANOVA for their significance and p-values and adjusted p-values with Benjamini-Hochberg correction are stated in this table.

Most peaks were only significant for a “raw” $p < 0.05$ rather than for an adjusted p -value. Usually the adjusted p -value is used to diminish the false-positive rate due to multiple testing. In metabolomic analyses, this can be restrictive as several peaks can be due to one metabolite and typically, when 280 or more variables are present this leads to a rather conservative adjustment. A thorough annotation of metabolites and assignment of many peaks would therefore decrease the chance to identify differences. For this reason, for all following results only the p -value was used as a significance level and the adjusted p -value will be stated only.

From PCA (**Figure 3.2**) and PLS-DA (**Figure 3.3**) the JIA group showed a higher variance than the other two groups. Clear differences between JIA and JSLE and healthy controls were noted, and the higher variance within the JIA patient group would indicate potential differences between JIA sub-groups themselves. Further analyses of the JIA serum samples are therefore warranted, but beyond the scope of this present analysis.

For going forward therefore, in order to reduce the variance and focus specifically on the characteristics of the metabolome in JSLE, JSLE patient samples were compared directly against the healthy control group only.

3.5.1.2 Differences between the serum metabolome of JSLE patients and of healthy paediatric controls

JSLE is a disease with many different manifestations from skin involvement to joint damage or nephritis. Patients may receive medication with higher and lower concentrations of DMARDs, steroids or immunosuppressants, but also other treatments like biologics. All these variables are explanations for a high variance to be observed within the JSLE cohort. PCA with PC1 and PC2 for JSLE and control patients gave 64.9% of the overall variance but leaving patient “L301” as an outlier (**Figure 3.4 A**). As **Figure 3.4 (B)** shows, PC1 and PC3 are still accounting for 62.3% of the overall variance and “L301” is not an outlier anymore. Therefore, all samples were considered for further analysis. PLS-DA created a more robust ($R^2=0.95$) and more predictive ($Q^2=0.34$) model (**C**) than

when comparing JIA, JSLE and control patients (**Figure 3.3**). Metabolites, e.g. Arginine, myo-Inositol and Hypoxanthine, are influential on component 1 and listed in the VIP score (**Figure 3.4D**).

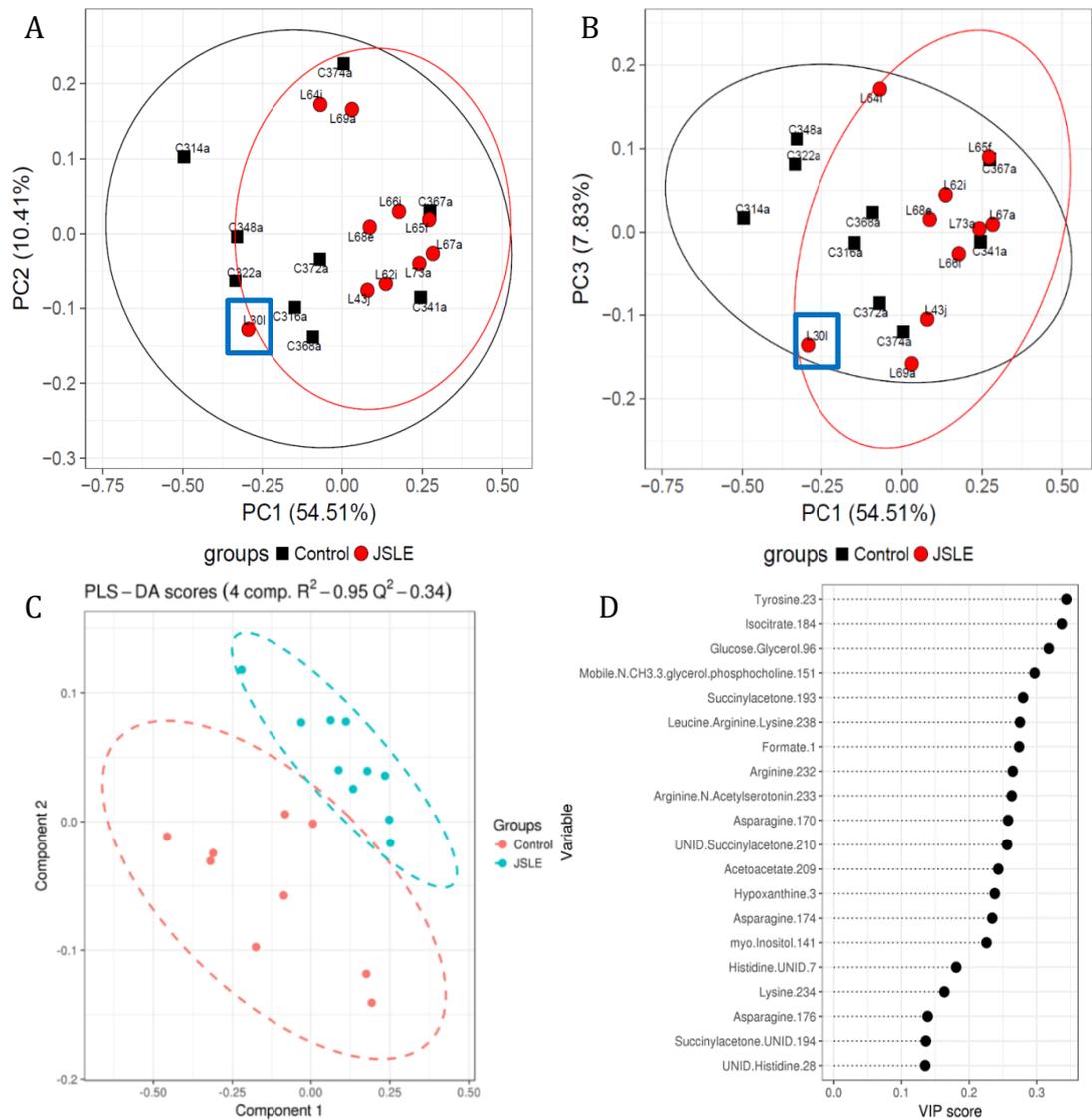


Figure 3.4: PCA and PLS-DA of metabolites present in JSLE and control serum. PCA of JSLE (red circles, n=10) and control (black squares, n=9) serum for PC1 and 2 explaining 64.9% of the overall variance in (A) indicated that patient L30l (indicated in blue square) is an outlier. PC2 and 3 in (B) make up 62.3% and include L30l which is included in analysis. A separation of the two groups with PLS-DA can be visualized with two components (C) but the best model is achieved with four components. The robustness of the test reaches an R^2 of 0.95 and the predictive power Q^2 is 0.34 with the VIP score of the most influential metabolites listed in (D).

Significant difference between disease and healthy controls was observed with a Kolmogorov–Smirnov test assuming non-parametric distribution and unequal variance in the two groups

Table 3.5). Due to small sample size a parametric distribution and equal variance could not be proved. The peaks are listed from lowest to highest p-value.

Metabolites	p-values	BH adjusted p-values
Mannose.81	0.0027	0.18
N.Acetylserotonin.Phenylalanine.14	0.0037	0.18
UNID.Glutamine.188	0.007	0.18
Ornithine.160	0.012	0.18
Asparagine.169	0.018	0.18
mobile_unsaturated_lipids.42	0.018	0.18
myo.Inositol.139	0.018	0.18
N.Acetylserotonin.15	0.018	0.18
Propylene_glycol.261	0.018	0.18
UNID.Valine.212	0.018	0.18
UNID.Valine.213	0.018	0.18
Glutamine.UNID.219	0.021	0.18
Histidine.Arabinose.72	0.021	0.18
UNID.Glutamine.223	0.021	0.18
Creatine_phosphate.79	0.045	0.31
Leucine.269	0.045	0.31

Table 3.5: Statistical analysis using Kolmogorov-Smirnov-test to assess difference between healthy paediatric control and JSLE serum metabolites. P-values are stated as well as Benjamini-Hochberg (BH) adjusted p-values.

PCA as seen in **Figure 3.4 A** indicates high variation within both controls and the JSLE cohort. Our group has previously obtained transcriptomics data from patients whose sera were used in this metabolomics analysis. Grouping samples into “Control”, “IFN high” and “IFN low” resulted in tighter clustering for the two JSLE subgroups (**Figure 3.5A**). These subgroups still indicated significant differences with ANOVA as seen in **Table 3.6**. For Asparagine, N-Acetylserotonin and Ornithine even several peaks are coming up as significant supporting that the observed results are true.

PCA of IFN high, IFN low and control patients (**Figure 3.5A**) displays a compact confidence interval with no outliers for IFN low patients strongly supporting the hypothesis that IFN low and IFN high patients are different subgroups of JSLE. Also, for IFN high patients a smaller confidence interval can be reached for PCA (**A**, red circles n=7) compared to the PCA obtained separating JSLE against control patients (**Figure 3.4**). This is further visualized with a PLS-DA which separates IFN low patients from both IFN high and control patients (**Figure 3.5B**). Even though the robustness R^2 is reaching values up to 0.99 this model does not reach a positive predictive power. PLS-DA diagnostics show R^2 and Q^2 for one to eight components (**C**). It shows that separation is very robust with increased number of components (R^2 reaching up to 0.99), but that predictive power remains very low with even negative values. Our analysis only included three IFN low patients, a positive predictive power is therefore not likely, and this model needs a larger cohort for effective validation ($n \geq 6$ required before PLS-DA can be effective). For this reason, metabolites responsible for PLS-DA separation were not considered for further analysis.

Metabolites	p-value	BH adjusted p-value	Different between
Acetoacetate.209	0.030	0.36	IFN low-IFN high
Asparagine.169	0.036	0.36	IFN low-Control
Asparagine.174	0.020	0.34	IFN low-IFN high
Asparagine.176	0.048	0.40	IFN low-IFN high
Glutamine.UNID.219	0.012	0.24	IFN high-Control, IFN low-Control
Histidine.Arabinose.72	0.0058	0.17	IFN high-Control
Leucine.Isoleucine.UNID.271	0.035	0.36	IFN high-Control
Mannose.81	0.0099	0.24	IFN high-Control, IFN low-Control
N-Acetylserotonin.15	0.0058	0.17	IFN high-Control, IFN low-Control
N-Acetylserotonin. Phenylalanine.14	0.0014	0.08	IFN high-Control, IFN low-Control
Ornithine.158	0.028	0.36	IFN high-Control
Ornithine.160	0.039	0.36	IFN high-Control n.s. p=0.06
UNID.Glutamine.188	0.00084	0.082	IFN high-Control, IFN low-Control
UNID.Histamine.140	0.039	0.36	IFN low-IFN high

Table 3.6: Results of ANOVA for serum metabolites which are significantly different between the patient groups Control, IFN low and IFN high. P-values are stated as well as Benjamini-Hochberg (BH) adjusted p-values. n.s. (not significant). Groups are stated different if $p < 0.05$ for Tukey's *post hoc* analysis.

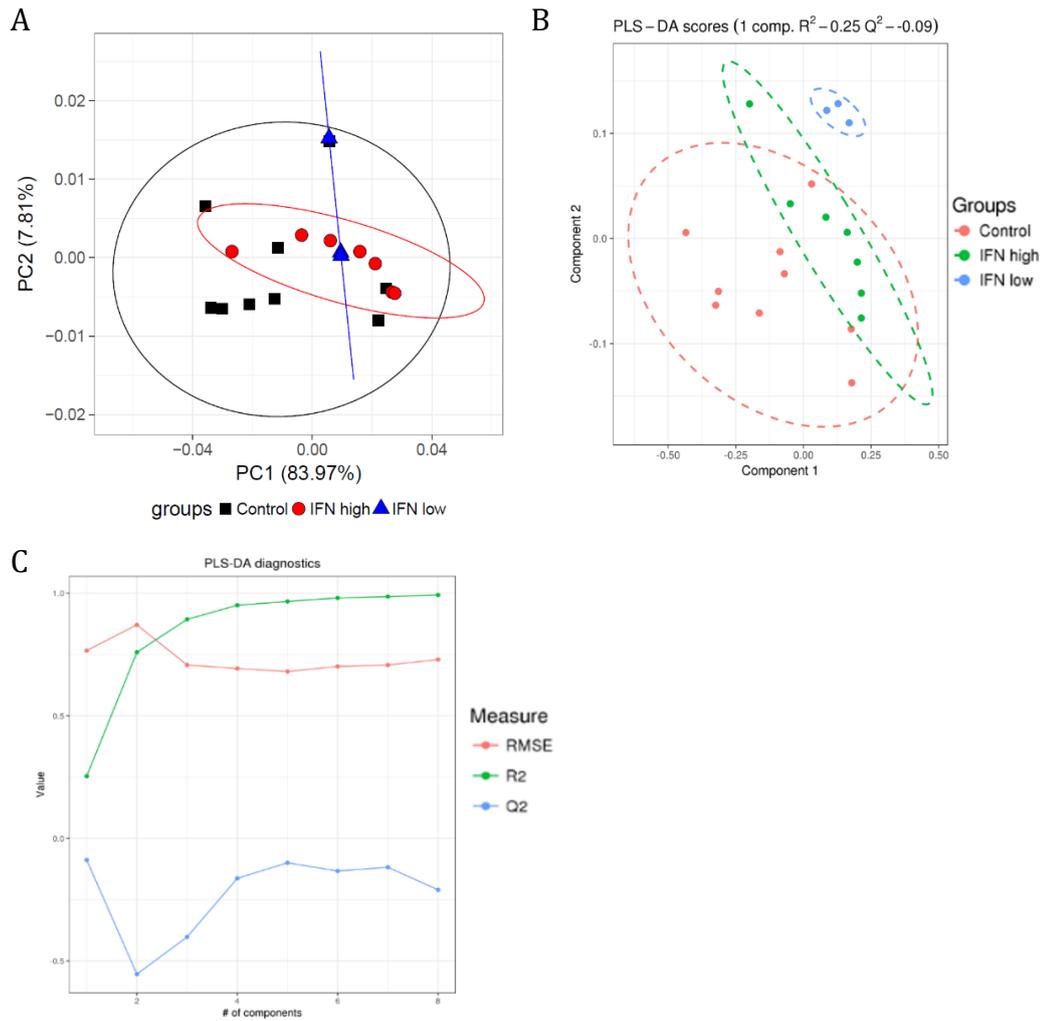


Figure 3.5: PCA and PLS-DA of serum metabolites of JSLE IFN high and low and control patients. PCA in (A) shows that PC1 summarises 84.0% of the variance within the samples and that PC1 and PC2 together reach over 90%. IFN low patients (blue, $n=3$) cluster closely together in both PCA (A) and PLS-DA (B). PLS-DA diagnostics (C) show that separation is very robust with increased number of patients (R^2 reaching up to 0.99), but that predictive power remains very low with negative values indicative of no predictive capabilities.

3.5.2 Urine metabolomics

3.5.2.1 Differences between the urine metabolome of JSLE patients, JIA patients and healthy paediatric controls

PCA of urine, similar to serum, did not show separation of JSLE, JIA and healthy paediatric control patients and PC1 together with PC2 only explained 55.9% of the variance (**Figure 3.6A**). Furthermore, no outliers were observed for any of the groups. Using PLS-DA of urine metabolites (**B**) as a model only two components were needed to reach a robustness of 0.91 and a predictive power of 0.41. Additionally, ANOVA showed significant differences of metabolites between the three groups. The main differences were between JSLE and JIA including Allantoin, Glucarate, Malate and Phenylalanine (**Table 3.7**).

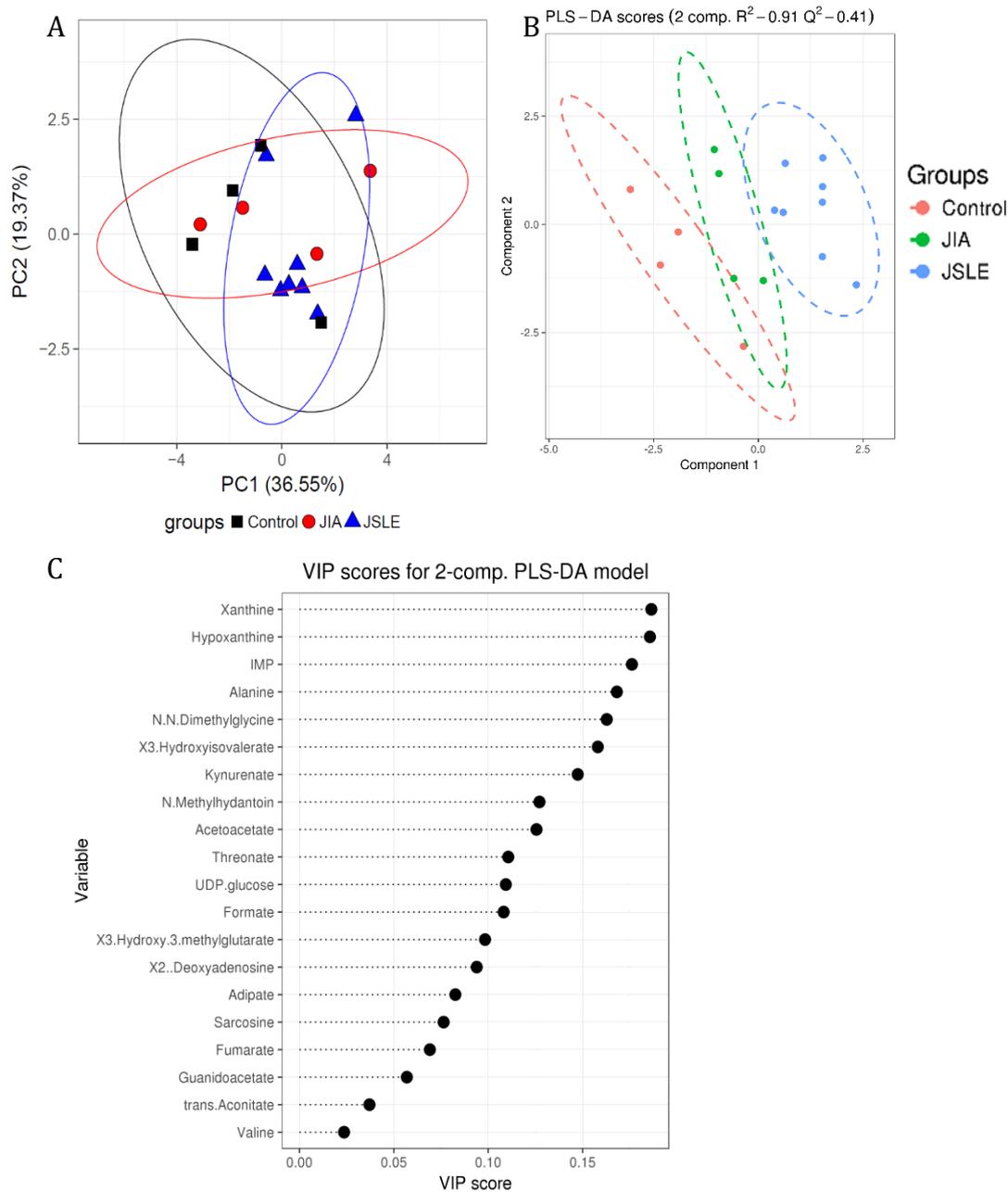


Figure 3.6: PCA and PLS-DA of urine metabolites of JSLE, JIA and healthy control patients. Patients were evaluated with unsupervised analysis (A) dependent on variance within samples. Control (black squares, $n=4$), JIA (red circles, $n=4$) and JSLE patients (blue triangles, $n=8$) do not separate when looking at 55.9% overall variance with PC1 and PC2. A robust ($R^2=0.91$) separation of Control (red), JIA (green) and JSLE (blue) patients can be reached with a model of two components (B) which achieves a predictive power (Q^2) of 0.41. Variables contributing to these Component 1 and 2 are displayed in (C).

Metabolites	p-value	BH adjusted p-value	Different between
3,4-Dihydroxybenzeneacetate	0.015	0.72	JIA-Control, JSLE-Control
3-Phenylpropionate	0.013	0.72	JSLE-Control, JSLE-JIA
Allantoin	0.039	0.72	JSLE-JIA
Glucarate	0.023	0.72	JSLE-JIA
Malate	0.0059	0.72	JSLE-JIA
Phenylalanine	0.036	0.72	JSLE-JIA

Table 3.7: Urinary metabolites showing a significant difference with ANOVA between JSLE, JIA and control patients. Adjusted p-values calculated with Benjamini-Hochberg (BH) are stated besides p-values and groups with differences after Tukey's *post hoc* analysis are listed in the last column.

Similar to serum samples also urine metabolites result in the overlap of confidence intervals of JIA and control patients in the PLS-D analysis (**Figure 3.5B**). Differences of ANOVA are mainly seen between JSLE and JIA patients.

A comparison of JSLE and control patients resulted in a better model between disease and healthy controls when serum metabolites were compared (as described in Section 3.5.1.2). For these reasons, analysis with only these two groups was also conducted for urine samples.

3.5.2.2 Differences between the urine metabolome of JSLE patients and of healthy paediatric controls

PCA for urine metabolites of JSLE and healthy paediatric control patients did not show outliers but a high overlap of the 95%-confidence intervals of JSLE and control patients (**Figure 3.7A**). With PLS-DA a model with a clear separation was achieved with two components and a R^2 of 0.92 and a Q^2 of 0.28 (**B**). Metabolites influencing this model are shown in the VIP score (**C**). The accuracy of the model meaning its robustness and predictive power are comparable to the model obtained from serum with R^2 of 0.95 and Q^2 of 0.34. This is somewhat surprising to see as the urine sample size ($n=12$) was smaller compared to serum ($n=19$).

Further separation of JSLE patients into IFN high and IFN low is difficult as in the cohort studied only two patients who donated urine were IFN low patients. Nevertheless, a PCA **Figure 3.8** of these three groups was undertaken and PC1 and PC2 covered 46.5% of variance. While there were no outliers observed with PCA, a separation between IFN high and low patients was clearly visible already with this unsupervised analysis. Isolation of IFN low patients resulted in a tighter confidence interval. PLS-DA at this stage was not possible with $n=2$ for IFN low patients. Interestingly, comparing this PCA with the PCA in **Figure 3.7** there was a similar pattern with two JSLE samples being further away from the other 6. The samples used for this separation included an additional sample of L34h. When labelling the samples with their study number the two IFN low patients indicated with a blue square were separated further away from all other patients (**Figure 3.8B**). The additional sample of L34h is located in the middle of the cluster of IFN high patients. Consequently, it seems likely that this patient is an IFN high patient which raises the possibility of using PCA of urine metabolites as a tool for differentiation of IFN high and low patients.

With this striking separation it is crucial to get information about differences in urine and as PLS-DA could not be performed and only results from ANOVA were available. These results need to be treated with caution due to the small sample size particularly with in the IFN low group. It is possible to calculate a p-value for a group containing only two samples, but importance in a wider cohort

would need to be validated. Metabolites are listed in **Table 3.8**. The metabolites in this table are mainly different between IFN high and IFN low, including Formate, 2-Hydroxyisobutyrate, Uridine-diphosphate (UDP) glucose, Valine and Acetate. Sarcosine, Sucrose and Fumarate are examples of metabolites being different for IFN high and IFN lows and also IFN low and control patients which is supporting the division between IFN low and IFN high patients seen in **Figure 3.8A**.

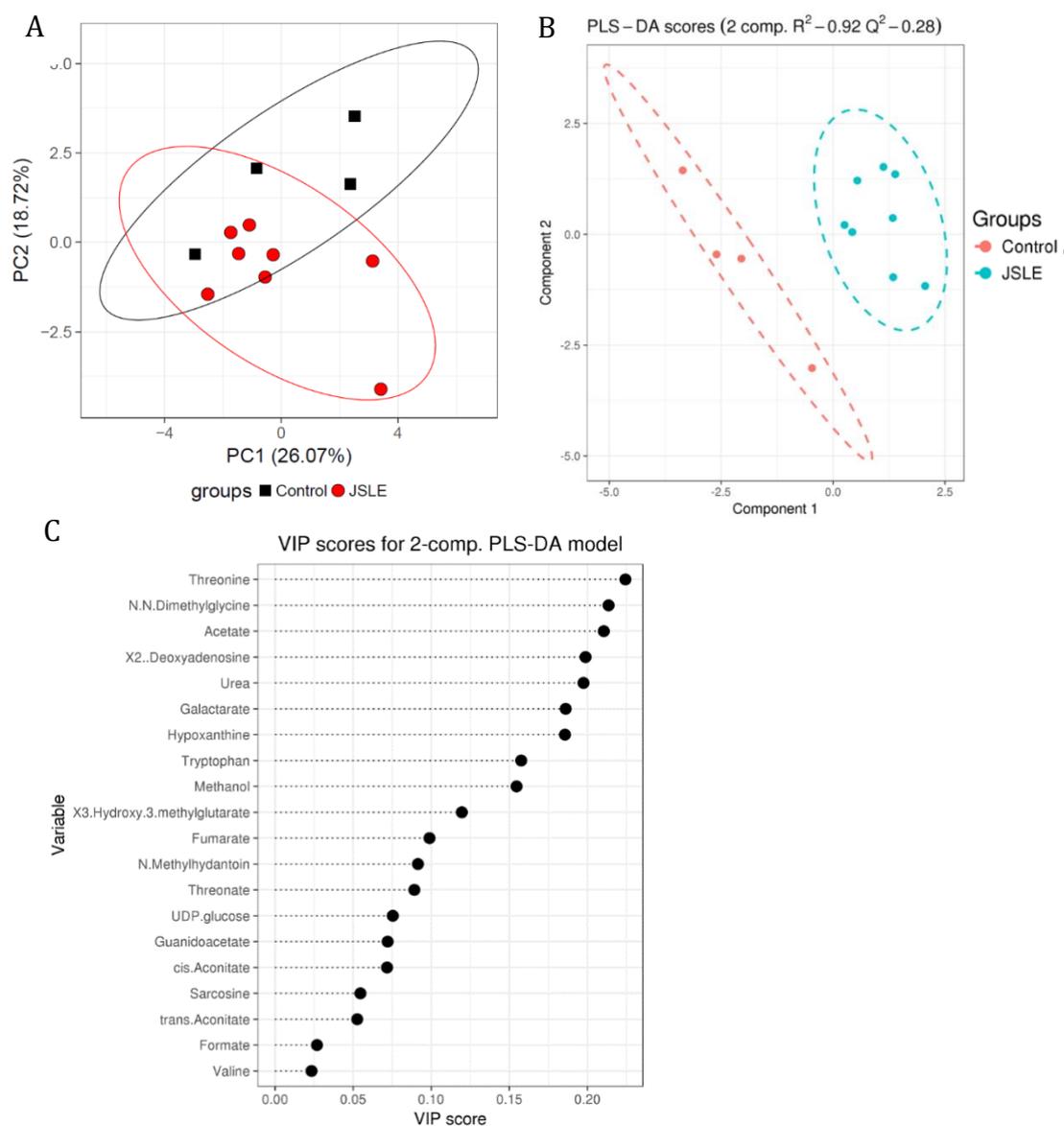


Figure 3.7: PCA and PLS-DA of metabolites present in JSLE and control urine. PCA of JSLE (red circles, $n=9$) and control (black squares, $n=4$) urine for PC1 and 2 explaining 44.8% of the overall variance in (A) did not indicate outliers. A separation of the two groups with PLS-DA can be visualized with two components (B) which is also the most accurate model with a robustness of R^2 of 0.92 Q^2 of 0.28 with the VIP score of the most influential metabolites displayed in (C).

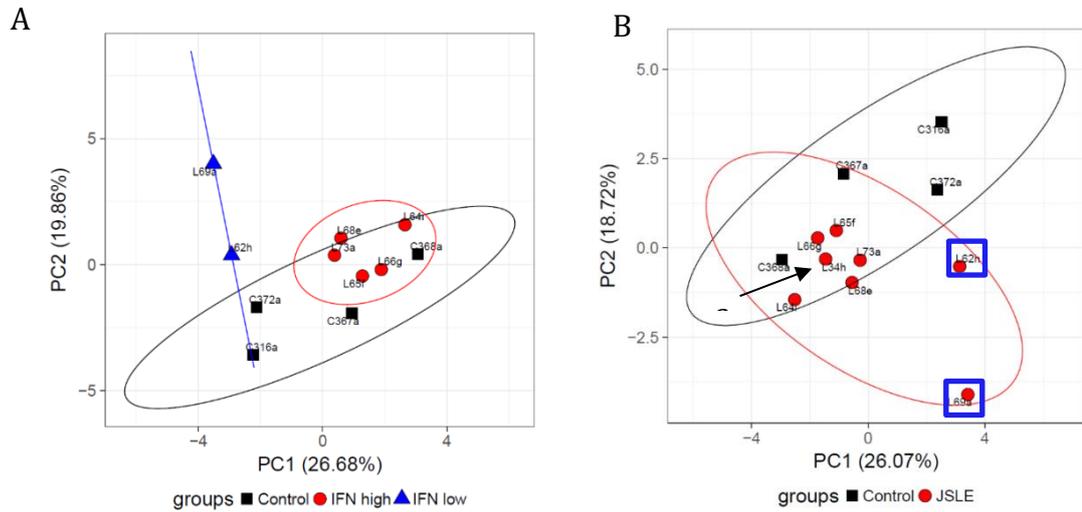


Figure 3.8: PCA of urine metabolites of IFN high, IFN low and control patients and of JSLE and control patients with indication of IFN subgroup. PC1 and PC2 together made up 46.5% of variance and separated the two disease subgroups of IFN low patients (blue triangles, n=2) and IFN high patients (red circles, n=5) in (A). Unsupervised analysis of JSLE (red circles, n=8) and control patients (black squares, n=4) in (B) revealed that IFN low patients indicated with blue squares do not fall within IFN high patients. L34h (black arrow) was not part of the transcriptomics study and is therefore not classified as IFN high or low, but the sample falls within the IFN high patient group.

Metabolite	p-value	BH adjusted p-value	Different between
2-Hydroxyisobutyrate	0.035	0.37	IFN low-IFN high
2-Oxoglutarate	0.047	0.37	n.s. (0.052 for IFN low -Control)
3,4-Dihydroxybenzeneacetate	0.024	0.37	IFN high-Control
3-Hydroxy-3-methylglutarate	0.0086	0.21	IFN low-Control, IFN low-IFN high
Acetate	0.011	0.23	IFN low-Control, IFN low-IFN high
cis-Aconitate	0.0015	0.11	IFN high-Control, IFN low-Control, IFN low-IFN high
Erythritol	0.0069	0.20	IFN high-Control, IFN low-Control, IFN low-IFN high
Ethanolamine	0.045	0.37	IFN low-Control
Formate	0.038	0.37	IFN low-IFN high
Fumarate	0.033	0.37	IFN low-Control, IFN low-IFN high
Glycolate	0.032	0.37	IFN low-IFN high
Leucine	0.045	0.37	IFN low-IFN high
Mannitol	0.0040	0.15	IFN low-Control, IFN low-IFN high
Pyruvate	0.025	0.37	IFN low-Control, IFN low-IFN high
Sarcosine	0.0038	0.15	IFN low-Control, IFN low-IFN high
Sucrose	3.03E-07	4.46E-05	IFN low-Control, IFN low-IFN high
UDP-glucose	0.040	0.37	IFN low-IFN high
Valine	0.021	0.37	IFN low-IFN high

Table 3.8: Urine metabolites showing a significant difference with ANOVA between JSLE IFN high, IFN low and control patients. Both the p-value and the Benjamini-Hochberg (BH) adjusted p-values are stated. n.s. (not significant). Groups are stated different if $p < 0.05$ for Tukey's *post hoc* analysis.

3.5.3 Pathway analysis

PCA and PLS-DA as well as ANOVA indicated strong distinctions between the two diseases JSLE and JIA compared to control patients for both serum and urine. JSLE IFN high and IFN low subgroups were only described based on their genetic signature so far and consequently pathway analysis with these groups can show additional differences.

Observed differences need to be looked at from a biological perspective and hence pathway analysis of the data was conducted. For this, metabolites with a significant p-value and the ten metabolites most influential on the VIP score were used.

3.5.3.1 Pathways important for JSLE, JIA and control patients

Metabolites that were included for analysis are listed in **Table 3.9** with their number for the human metabolome database (HMDB), PubChem database and Kyoto Encyclopaedia of Genes and Genome database (KEGG).

Query	Match	HMDB	PubChem	KEGG
3,4-Dihydroxy-benzeneacetate	3,4-Dihydroxy-benzeneacetic acid	01336	547	C01161
3-Hydroxyiso-valerate	3-Hydroxyisovaleric acid	00754	69362	NA
3-Phenylpropionate	Hydrocinnamic acid	00764	107	C05629
Acetoacetate	Acetoacetic acid	00060	96	C00164
Alanine	L-Alanine	00161	5950	C00041
Allantoin	Allantoin	00462	204	C01551
Arginine	L-Arginine	00517	6322	C00062
Asparagine	L-Asparagine	00168	6267	C00152
Creatine-phosphate	Phosphocreatine	01511	587	C02305
Dimethylglycine	Dimethylglycine	00092	673	C01026
Formate	Formic acid	00142	284	C00058
Glucarate	Glucaric acid	00663	33037	C00818
Glucose	D-Glucose	00122	5793	C00031
Glycerol	Glycerol	00131	753	C00116
Hypoxanthine	Hypoxanthine	00157	790	C00262
IMP	Inosinic acid	00175	8582	C00130
Kynurenate	Kynurenic acid	00715	3845	C01717
Lysine	L-Lysine	00182	5962	C00047
Malic acid	L-Malic acid	00156	222656	C00149
Mannose	D-Mannose	00169	18950	C00159
Mobile lipids	NA	NA	NA	NA
N-Acetylserotonin	N-Acetylserotonin	01238	903	C00978
N-Methylhydantoin	N-Methylhydantoin	03646	69217	C02565
Ornithine	Ornithine	00214	6262	C00077
Phenylalanine	L-Phenylalanine	00159	6140	C00079
Succinylacetone	Succinylacetone	00635	5312	NA
Threonate	Threonic acid	00943	151152	C01620
Xanthine	Xanthine	00292	1188	C00385

Table 3.9: List of metabolites included in pathway analysis of JIA, JSLE and control patients. Results from ANOVA as well as from PLS-DA VIP scores were integrated. Metabolites were inserted into Metaboanalyst pathway analysis with the indicated HMDB, PubChem and KEGG number. Lipids are not included in this type of analysis and therefore not recognized by the program and marked as not available (NA).

Metaboanalyst pathway analysis yielded 35 pathway “hits”, but only seven of them had a significant p-value with Fisher’s exact test and are therefore stated here. These pathways included Aminoacyl-tRNA biosynthesis, arginine and ornithine metabolism, nitrogen metabolism, arginine and proline metabolism, galactose metabolism, alanine, aspartate and glutamate metabolism and pyruvate metabolism (see **Table 3.10**).

Pathway	Matched metabolites	Total number of metabolites	p-value (Fisher's exact test)
Aminoacyl-tRNA biosynthesis	<i>L-Asparagine, L-Phenylalanine, L-Arginine, L-Lysine, L-Alanine</i>	75	8.38E-04
D-Arginine and D-ornithine metabolism	<i>L-Arginine, Ornithine</i>	8	0.0027918
Nitrogen metabolism	<i>L-Phenylalanine, L-Asparagine, Formic acid</i>	39	0.007071
Arginine and proline metabolism	<i>L-Arginine, Ornithine, Phosphocreatine, N-Methylhydantoin</i>	77	0.0073479
Galactose metabolism	<i>D-Glucose, Glycerol, D-Mannose</i>	41	0.0081356
Alanine, aspartate and glutamate metabolism	<i>L-Asparagine, L-Arginine</i>	24	0.02486
Pyruvate metabolism	<i>L-Malic acid, Formic acid</i>	32	0.042476

Table 3.10: List of significant pathways for metabolites of JIA, JSLE and control patients. Ten most influential metabolites of the VIP score for PLS-DA and significant metabolites of ANOVA were used in metaboanalyst to identify pathways. Fisher's exact test was used to identify significance.

Combining these pathways and further investigation of the role of these metabolites indicated a potential biological role of these metabolites in JSLE and JIA patients. These metabolites are summarized in a pathway containing glycolysis, Pentose phosphate pathway (PPP), Krebs and urea cycle as shown in **Figure 3.9**. Differences in these pathways for JSLE and JIA patients is summarized in **Figure 3.10**. Both the PPP as well as the Krebs cycle appear to be fed by glucose in JSLE patients with a preference for the PPP which is increased compared to healthy control patients. JIA patients do not have increased glucose levels and appear to use it only for the PPP indicated by high IMP and xanthine levels as observed for JSLE patients. Metabolites of the Krebs cycle are increased for JIA patients but appear normal in JSLE. The increased need of energy in the rheumatic patients is satisfied with input of acetoacetate from triacylglyceride and acetoacetate and fumaric acid resulting from phenylalanine degradation. Arginine is increased in both disease groups and with it, ornithine. This observation is supported by higher guanidinoacetate levels resulting from ornithine production. Glutamine synthesis is increased in JIA patients, but inhibited in JSLE patients due to higher concentrations of alanine. Glutamine is also feeding into the urea cycle which seems upregulated in both diseases.

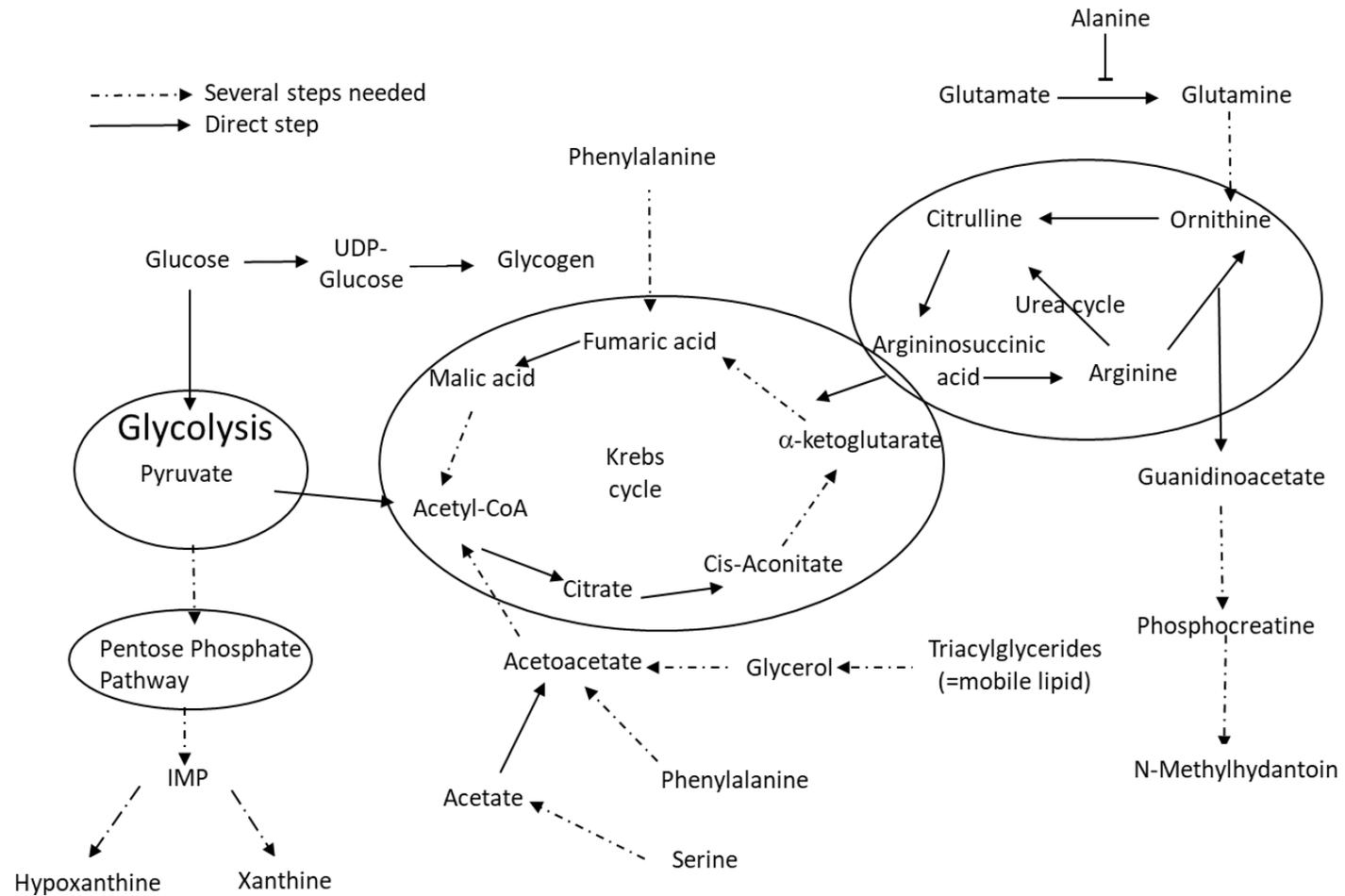


Figure 3.9: Connections between metabolic pathways potentially important for autoimmune diseases. Glycolysis, the Pentose Phosphate Pathway, the Krebs cycle and the Urea cycle are all connected, and metabolites feeding into this pathway or being released from them are shown here.

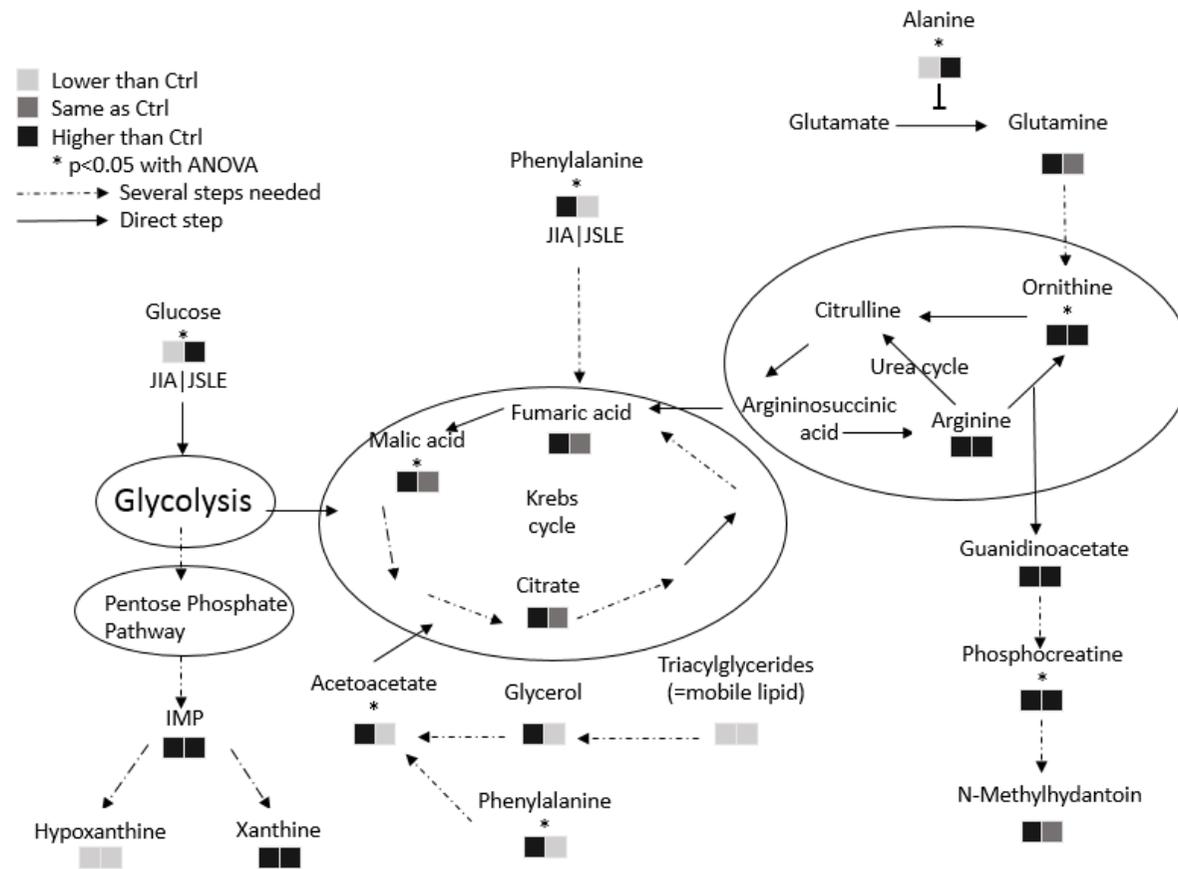


Figure 3.10: Altered metabolic pathway in JIA and JSLE patients compared to control. The pathway includes Glycolysis, Pentose Phosphate Pathway, Krebs cycle, and the Urea cycle with their starting and end products. Metabolites that were detected either in serum or urine are shown with squares for JIA on the left and JSLE on the right, under the name of the metabolites. Concentrations compared to control are indicated with ■ for higher than control, ▒ similar to control and □ for lower than control. If a metabolite was significantly different with ANOVA ($p < 0.05$) then this is indicated with *

3.5.3.2 Pathways important for JSLE IFN low, JSLE IFN high and healthy control patients

Sections 3.5.1.2 and 3.5.2.2 described differences in serum and urine metabolites between subsets of JSLE patients which were suggested to be separable by their IFN signature. JSLE IFN low patients are very rare and PLS-DA for serum showed a negative result for Q^2 which is again due to the low number in the experimental sample. For urine, PLS-DA could not be conducted due to the low numbers. Therefore, for pathway analysis only metabolites which were significantly different with ANOVA were inserted into Metaboanalyst.

Metabolites used for analysis are listed in **Table 3.11** with their HMDB, PubChem and KEGG ID. The resulting pathways are stated in **Table 3.12**.

Metabolite	HMDB	PubChem	KEGG
3,4-Dihydroxybenzeneacetic acid	01336	547	C01161
3-Hydroxymethylglutaric acid	00355	1662	C03761
Acetic acid	00042	176	C00033
Acetoacetic acid	00060	96	C00164
Alpha-Hydroxyisobutyric acid	00729	11671	NA
cis-Aconitic acid	00072	643757	C00417
D-Mannose	00169	18950	C00159
Erythritol	02994	222285	C00503
Ethanolamine	00149	700	C00189
Formic acid	00142	284	C00058
Fumaric acid	00134	444972	C00122
Glycolic acid	00115	757	C00160
L-Asparagine	00168	6267	C00152
L-Leucine	00687	6106	C00123
L-Valine	00883	6287	C00183
Mannitol	00765	6251	C00392
N-Acetylserotonin	01238	903	C00978
Ornithine	00214	6262	C00077
Oxoglutaric acid= α -ketoglutarate	00208	51	C00026
Pyruvic acid	00243	1060	C00022
Sarcosine	00271	1088	C00213
Sucrose	00258	5988	C00089
Uridine diphosphate glucose	00286	53477679	C00029

Table 3.11: Serum and urine metabolites which were significantly different between IFN high, IFN low and control patients.

<i>Pathway</i>	<i>Matched metabolites</i>	<i>Metabolites</i>	<i>p-value</i>
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		(total)	(Fisher's exact test)
Citrate cycle (TCA cycle)	Oxoglutaric acid, cis-Aconitic acid, Pyruvic acid, Fumaric acid	20	2.3073E-5
Alanine, aspartate and glutamate metabolism	L-Asparagine, Pyruvic acid, Oxoglutaric acid, Fumaric acid	24	4.9399E-5
Glyoxylate and dicarboxylate metabolism	cis-Aconitic acid, Oxoglutaric acid, Formic acid, Glycolic acid, Pyruvic acid	50	6.3699E-5
Butanoate metabolism	Acetoacetic acid, Pyruvic acid, Oxoglutaric acid, Fumaric acid	40	3.8582E-4
Valine, leucine and isoleucine biosynthesis	Pyruvic acid, L-Leucine, L-Valine	27	0.0016827
Pyruvate metabolism	Pyruvic acid, Formic acid, Acetic acid,	32	0.0027699
Tyrosine metabolism	3,4-Dihydroxy- benzeneacetic acid, Pyruvic acid, Fumaric acid, Acetoacetic acid	76	0.0043584
Arginine and proline metabolism	Ornithine; Fumaric acid Pyruvic acid Sarcosine	77	0.0045695
Valine, leucine and isoleucine degradation	L-Leucine, Acetoacetic acid, L-Valine	40	0.0052619
Galactose metabolism	Sucrose, UDP- glucose, D-Mannose	41	0.0056437
Ascorbate and aldarate metabolism	UDP-glucose, Pyruvic acid, Oxoglutaric acid	45	0.0073367
Taurine and hypotaurine metabolism	Pyruvic acid, Acetic acid	20	0.013719
Pantothenate and CoA	L-Valine, Pyruvic acid	27	0.024384

biosynthesis			
Aminoacyl-tRNA biosynthesis	L-Asparagine, L-Valine, L-Leucine	75	0.029237
Glycolysis or Gluconeogenesis	Acetic acid, Pyruvic acid	31	0.031601
Vitamin B6 metabolism	Oxoglutaric acid, Pyruvic acid	32	0.033523
Propanoate metabolism	Acetoacetic acid, L-Valine	35	0.039557
Nitrogen metabolism	L-Asparagine, Formic acid	39	0.048196

Table 3.12: List of significant pathways for metabolites of JIA, JSLE and control patients. Ten most influential metabolites of the VIP score for PLS-DA and significant metabolites of ANOVA were used in metaboanalyst to identify pathways. Fisher's exact test was used to identify significance.

Some metabolites including acetoacetic acid, formic acid or ornithine, are observed for both JIA and JSLE patients when comparing them to paediatric healthy controls. However, many of the other significant metabolites between JSLE subsets and healthy control patients differed to the JIA, JSLE and control patient comparison shown in **Figure 3.10**.

The connections were therefore adjusted to the metabolites for JSLE IFN high, IFN low and control patients in **Figure 3.11**. The PPP shunt was visible in both JSLE subgroups, but otherwise they strongly differed. The Krebs cycle appears to be highly active in IFN low patients with high glucose and pyruvate, but also acetoacetate supporting energy production. Ornithine is increased in both IFN high and IFN low patients, but only IFN high patients have high glutamine levels. IFN high patients seem to utilize arginine for citrulline production and not for ornithine synthesis as the by-product guanidinoacetate is decreased. Ornithine in JSLE IFN high patients might result rather from glutamine and could then result in citrulline production. This cannot be confirmed with $^1\text{H-NMR}$ of serum and urine as these liquids have too many strong signals from high concentration metabolites which could mask signals from citrulline.

Overall the IFN low patients have a very different metabolic signature compared to IFN high patients and presented pathways are upregulated compared to controls.

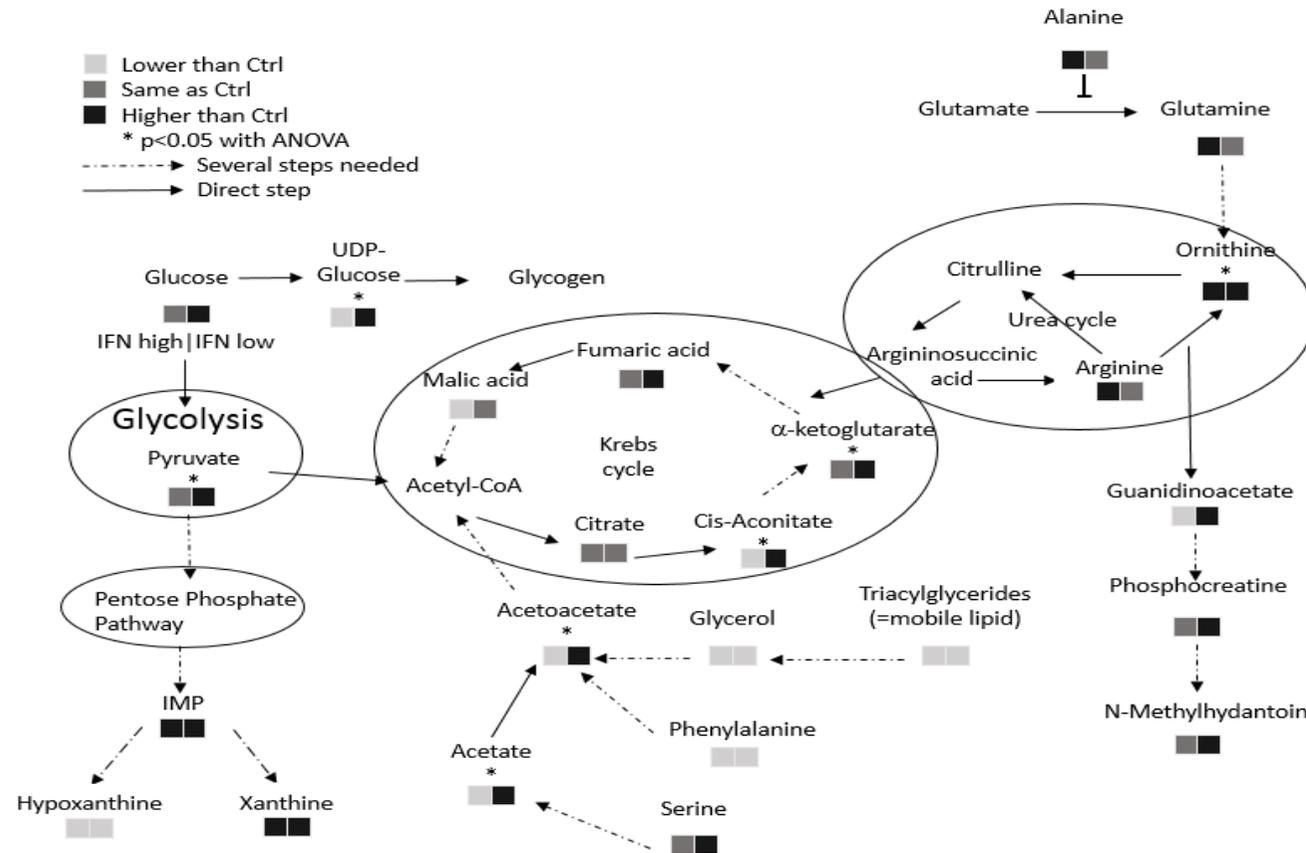


Figure 3.11: Altered metabolic pathway in JSLE patients with IFN high and IFN low signature compared to control. The pathway includes Glycolysis, Pentose Phosphate Pathway, Krebs cycle, and the Urea cycle with their starting and end products. Metabolites that were detected either in serum or urine are shown with squares for JIA on the left and JSLE on the right, under the name of the metabolites. Concentrations compared to control are indicated with ■ for higher than control, ■ similar to control and ■ for lower than control. If a metabolite was significantly different with ANOVA ($p < 0.05$) then this is indicated with *.

3.6 Discussion

This study aimed to look at differences between metabolites in serum and urine of JIA, JSLE and healthy control patients. Additionally, an objective was to compare IFN high and IFN low JSLE patients who were characterised based on results of their IFN induced gene signature from previously obtained transcriptomics data. It was hypothesized that changes in metabolite profiles could be potentially used as diagnostic tools for disease groups and therefore wanted to test which body fluid would provide a better prediction model. Furthermore, metabolites and altered pathways might indicate dysregulations in a disease or a disease subgroup.

JSLE is a disease where many organs can be involved [53] and it is therefore called a multisystem disease. Most JIA subtypes are mainly affecting joints, but there is also a systemic form. The serum samples used for this study were solely from oligo- and polyarticular JIA subtype patients (**Table 3.1**). In the set of urine samples as seen in **Table 3.2** one JIA patient with systemic disease was included due to the limited sample numbers available for this study. PCA of JIA, JSLE and control patients showed (**Figure 3.6A**) that all samples including the urine of the systemic JIA patient fell within the 95% confidence interval and was hence not considered an outlier. The sample was consequently used for all analyses.

PCA for serum reached a higher percentage of variance adding PC1 and PC2 compared to PCA of urine metabolites. Several peaks were annotated for each metabolite in serum and change in one metabolite should change all or at least most peaks of this metabolite. Urine on the other hand only had one variable per metabolite due to analysis using Chemomx Profiler® as described in 3.4.3. When serum and urine were used to distinguish in a model with PLS-DA between JIA, JSLE and control patients' urine was found to be the more informative body fluid. While in serum a R^2 of 0.82 and a Q^2 0.18 was reached, urine achieved a R^2 0.91 R^2 and Q^2 0.41 (**Figure 3.3** and **Figure 3.6**) with a smaller sample size. A comparison of the JSLE subgroups was not possible since the sample size was too small to create a PLS-DA. Yet, these results suggest that urine is the more

informative body fluid to distinguish between JIA, JSLE and healthy controls. Studies for NMR techniques are often focused on serum even in diseases with kidney involvement which is expected to have more impact on the urine rather than the serum [85]. A reason for this could be that sample preparation for urine is more difficult than serum as it has to be buffered because of variation in the pH values [216]. Even with pre-treatment it is usually not possible to achieve equivalent pH between samples, and the acidity can alter the state of protonation of a metabolite like an amino acid which results in a different chemical shift [223]. For this reason, analysis by comparison of the area under peaks within certain ppm ranges was not possible. It was necessary that metabolites had to be annotated for each sample in Chenomx Profiler® which reduced the number of variants as each metabolite only resulted in one variant instead of several because of multiple peaks per metabolite. This might have influenced the ability of urine to be a good model builder, but due to the higher levels of intervention by the analyst, it may have limited use for large sample sets.

It has to be noted, that profiles obtained by these PLS-D analyses need to include all metabolites which contribute to Component 1 and Component 2 in order to cluster diseases or disease subtypes. The predictive power cannot be obtained for each single metabolite by this type of analysis and the VIP score only indicates which are the most influential metabolites. The overall predictive power of the PLS-DA models is the only predictive power which can be discussed. Due to the very small sample size the predictive power has been found low for most analyses in this thesis and all results need additional validation and need to be treated with caution. Furthermore, despite excluding unidentified metabolites, impact of medication on urine and serum may still be present in this analysis. A correlation analysis of all metabolites with the medication patients were treated with may find that some treatments affect certain metabolites. It cannot be distinguished though if these metabolites result from altered or less active disease or from processing medication. For a true understanding of impact of medication on serum and urine metabolite profiles a separate study with the same patients before and very shortly after

treatment would have to be conducted ideally with increasing doses. This however was beyond the scope of this project.

Using both body fluids enabled us to perform a more thorough pathway analysis as some metabolites were only detected in serum or only in urine. Metabolites important for PLS-DA or significantly different with ANOVA were included in initial pathway analysis and a more general overview was created afterwards in **Figure 3.10** for JIA, JSLE and healthy paediatric control and **Figure 3.11** JSLE IFN high, IFN low and healthy paediatric control comparison. The three major interactions were glycolysis, the PPP, Krebs and urea cycle.

For both JIA and JSLE IFN low patients the Krebs cycle showed increased activity as intermediates like α -ketoglutarate and fumarate were increased. These metabolites have been shown to support inflammation by regulating hypoxia-inducible factors (HIF) which are transcription factors ([224], [225]). HIF1 upregulates IL-1 β , a pro-inflammatory cytokine that is considered to play an important part in SLE [158].

When comparing JIA and JSLE patients, glucose levels showed a strong difference with JSLE patients seeming to be high in glycolysis using resulting products for both the PPP as well as the Krebs cycle. High glucose consumption has indeed been observed for example in CD4 cells from SLE patients for both naïve as well as activated T-cells, suggesting already an intrinsic effect [226].

JIA patients appear to have high acetoacetate levels which can support Krebs cycle activity. It has been suggested, that CD4-Tcells from RA patients use their glucose immediately for the PPP instead of for glycolysis which would support the observed need for ketone bodies to provide for the energy demands. In consequence ROS levels have been shown to be decreased in these cells [227]. Interestingly, in SLE T-cells mTORC1 activation was found leading to overreactivity of the PPP as observed in our JSLE cohort [228]. The PPP was found to be increased in all the three studied conditions which were JIA, JSLE IFN high and JSLE IFN low patients.

This pathways leads to nucleotide and NADPH production with the latter being a substrate for the NADPH oxidase [229]. The enzyme supports phagocytes in

ROS production and neutrophils additionally in NET production, suggesting importance of these cell types in disease [230].

Significance of phagocytes is further supported with mobile lipids and glycerol being low for JSLE as well as JIA patients. ¹H NMR is not able to distinguish well enough between fatty acids, therefore the previously described “dyslipoproteinemia” could not be confirmed [231]. Nevertheless, other studies showed decrease in glycerides in RA and SLE [232]. Phagocytes have been linked to the use of fatty acids as they have a high demand for lipids in order to generate membranes [233]. Future studies may additionally wish to employ orthogonal approaches such as mass spectroscopy to investigate differences in lipids. ¹H NMR has only very limited capabilities regarding detection of fatty acids and their distinction.

The third pathway linked to the investigated disease was the urea cycle. Also, here IFN low patients showed similarities to JIA patients with a higher ornithine production. IFN high patients seem to not use the conversion of arginine to ornithine, but instead a direct shunt to citrulline as products resulting from this conversion have been found low. Citrulline has been shown to be important in RA, NET production and is a common modification on lupus autoantigens [234].

Based on the comparison of their metabolite profile JSLE IFN low patients have many similarities to JIA patients. The majority of paediatric arthritis patients have been found to have an IFN low profile and the cause for the underlying signature could therefore be a strong contributor to the metabolic profile as well [56].

Analysis of different body fluids is not commonly used and may therefore need refinement for future use. Additionally, not all urine and serum samples were paired which would be a better approach to validate the ability to combine these data sets. Nevertheless, as metabolites were obtained by groups which did not have any outliers they are to some extent a homogenous group. Validity of these results is still not guaranteed and different disease activity status and medication of different episodes may influence results. Similar changes of the same metabolite in both serum and urine observed in our dataset for many

metabolites is yet a reassurance to some extent that the inclusion of the chosen samples is valid.

3.7 Conclusions

In conclusion this study has demonstrated that ^1H NMR is a useful tool to distinguish patients based on their metabolome into JIA, JSLE and paediatric control patients with urine being the optimal biofluid for separation. Separation of JSLE patients based on their IFN signature could not be evaluated due to the very small sample size. Nevertheless, pathway analysis revealed that metabolites from the Krebs cycle are increased which plays a vital role in inflammation. Further, the PPP was shown to be elevated in both diseases and both JSLE subgroups. In combination with glycerides being increased, this suggests increased production and use of NADPH, an invaluable substrate for phagocytes and especially in neutrophils for phagocytosis and NETosis. Their importance particularly in IFN high patients seems likely as the pathway analysis undertaken suggests that citrulline production is directly supported from arginine without ornithine as an intermediate. For most metabolites JSLE IFN low patients appear similar to JIA patients which could be due to the fact that JIA patients being more likely to be IFN low patients. Therefore, a specific comparison between JSLE IFN low and IFN high with focus on phagocytes might be an important approach to take forward.

4 IFN-induced and phagocytosis-related gene signature in JSLE neutrophils

4.1 Introduction

JSLE is a very heterogenous disease (see Section 1.3) with a wider variation in clinical characteristics but also potential biologically-defined disease subtypes based on their interferon gene signature (IGS) have been found and described [57]. Yet, little is known about why some patients have an IFN low and others an IFN high signature, and also about the differences between the IGS subgroups in the disease.

In Chapter 3 within the metabolomic analysis, differences between IFN low and IFN high JSLE patients were observed (albeit not demonstrating statistical significance, in part at least due to sample size limitations). Nevertheless, these results indicated important potential differences in pathways regulating phagocytosis between these groups.

Neutrophils are important for detection and phagocytosis of pathogens which is concluded with digestion in the phagosome and phagolysosome. Molecular patterns expressed on or by a microorganism can be directly recognized with pattern recognition receptors such as TLRs [180] and Dectin-1 [171] – see Section 1.7.1 and 1.7.2. Targets can also be opsonized with complement or antibodies and are then recognized by Fc- or complement receptors on the surface of neutrophils ([235], [236]) – see Section 1.7.5 and 1.7.6.

After uptake, the phagosome needs to fuse with the lysosome which involves changes of Ca^{2+} -levels by CamK1D [207] so that the enzymes from the cell can break down the pathogen (see Section 1.7.4). To improve phagocytosis efficiency neutrophils can release proteins such as S100A9 (see Section 1.7.7) to stimulate themselves in an autocrine manner [198].

It is considered that dysregulation in phagocytosis can lead to increased NETosis [171], a process found to cause cell and tissue damage in SLE patients [237] – see Section 1.6.4.2. As this type of cell death is specific to neutrophils

and thought to be a source of autoantigens it is essential to understand the ability of neutrophils to perform phagocytosis in JSLE.

Despite the potential importance of this mechanism in JSLE pathogenesis there is little research and understanding of this process in neutrophils of patients. To date, there are very limited data investigating the role of neutrophil phagocytosis in JSLE (see Section 1.6.5.2). Nevertheless, one study observed no significant impairment of phagocytosis of bacteria in neutrophils, but only against LPS derived from Salmonella [238]. Within the aforementioned study, there is no indication of the time points involved or whether serum from patients or controls were used. Therefore further investigation is required so that the effect of opsonisation and saturation of the neutrophils can be determined as this may play a part in why no difference was observed.

Data presented in **Chapter 3** have indicated that one of the key functions that may be important are the pathways regulating phagocytosis. Neutrophils from JSLE patients may have dysregulated phagocytosis based on both genetic as well as on a functional level, resulting in increased NET formation.

4.2 Chapter hypothesis

The hypothesis that will be investigated here is that JSLE IFN high and IFN low patients not only differ in their IFN-induced gene signature, but also differ in their phagocytosis-related gene signature.

4.3 Objectives

The specific objectives for this chapter are:

Objective 1: To determine an appropriate IGS to differentiate between JSLE IFN low and JSLE IFN high patients.

Objective 2: To measure the expression of genes that are involved in the modification of DNA and phagocytosis-related genes (PRGs) in neutrophils of JSLE patients with their respective IFN subtypes and healthy controls.

Objective 3: To compare protein expression of PRGs in JSLE neutrophils, and their respective IFN subtypes, and healthy paediatric control patient neutrophils.

Objective 4: To investigate the phagocytic ability of JSLE neutrophils compared to healthy control neutrophils.

4.4 Chapter specific methods

4.4.1 Real-time PCR using SYBR Green

For the Sections in this chapter cDNA and qPCR kits from Primerdesign were used unless stated otherwise. qPCR was performed as described in Sections 2.2.5.2 and 2.2.5.3. mRNA was obtained from neutrophils of healthy paediatric control patients and JSLE patients (see Section 2.1 and 2.2.5.1). Characteristics of both groups and the JSLE groups split into IFN high and low expressing patients are summarised below:

	<i>Control (n=13)</i>	<i>JSLE (n=13)</i>	<i>JSLE IFN high (n=10)</i>	<i>JSLE IFN low (n=3)</i>
Age	14.54±1.1	14.23±2.9	14.5±2.4	13.33±4.7
Female	62%	77%	80%	67%
WB	100%	62%	60%	66%
Medication	none	HCQ (9/13) Mycophenolate (8/13) Prednisolone (6/13) Azathioprine (1/13) Aspirin (1/13) Bisphosphonate (1/13) CP (2/13) ARB (1/13) Statin (1/13) no info (2/13)	HCQ (6/10) Mycophenolate (6/10) Prednisolone (3/10) Azathioprine (1/10) Aspirin (1/10) Bisphosphonate (1/10) CP (1/10) ARB (0/10) no info (2/10)	HCQ (3/3) Mycophenolate (2/3) Prednisolone (2/3) Azathioprine (0/3) Aspirin (0/3) Bisphosphonate (0/3) CP (2/3) ARB (1/3) no info (0/3)
Disease activity	ESR	13.1±13.8	15.6±14.36	3.0±2.8
	CRP	5.5±4.7	5.9±4.7	4.0±0.0
	C3	1.16±0.48	1.18±0.54	1.13±0.13
	C4	0.24±0.15	0.26±0.18	0.20±0.11
	dsDNA	19.10±50.9	27.23±58.0	0±0
	SLEDAI	6.17±5.9	4.22±5.1	12.00±3.6
	Renal BILAG	A (1/13) C (1/13) D (4/13) E (6/13) no info (1/13)	C (0/10) D (4/10) E (5/10) no info (1/13)	A(1/3) C(1/3) D (0/3) E (1/3)

Table 4.1: Demographic and clinical data about patients whose blood was used for real-time PCR. Abbreviations: White British ethnicity (%WB), Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Complement component 3 (C3), Complement component 4 (C4), anti-dsDNA titres (dsDNA), SLE Disease Activity Index (SLEDAI), British Isles Lupus Assessment Group (BILAG), Hydroxychloroquine (HCQ), Angiotensin II receptor blocker (ARB), Cyclophosphamide (CP)

4.4.2 Selection of housekeeping genes using GeNorm kit

The amount of cDNA added into a PCR reaction is quantified beforehand and should be equal between the tested samples, but normalization to housekeeping genes allows normalization of variations in starting material. In molecular biology, housekeeping genes are typically constitutive genes expressed in all cells of an organism under normal conditions and are generally required for the maintenance of basic cellular functions. Housekeeping genes however need to be selected carefully as even genes considered to be housekeeping genes may vary between treatment conditions or cell types.

The GeNorm™ reference gene selection kit from Primerdesign contains the following primers for testing:

18S, ACTB, ATP5B, B2M, CYC1, EIF4A2, GAPDH, RPL13A, SDHA, TOP1, UBC, YWHAZ.

qPCRs with six cDNA samples were performed with different conditions as for example neutrophil and PBMC cDNA samples or control and JSLE patients.

Results were then analysed with qbasePLUS (Biogazelle), which gives the M- and V-value as results. The M-value expresses variability of each gene between the conditions with a cut-off of 0.5 considered as stable. Genes are presented from the weakest (high M-value) to the strongest gene (lowest M-value) from left to right. The V-value determines how many housekeeping genes are required for optimal normalization with a cut-off of 0.15. Results are shown in step-wise inclusion of the next most stable gene.

Analysis revealed that optimal PCR of neutrophils and PBMCs requires four housekeeping genes, namely UBC, ACTB, TOP1 and GAPDH to reach a V-value of 0.15 which is shown in **Figure 4.1**.

In neutrophils from control and JSLE patients, M-values were lower showing that there is less variation (**Figure 4.2**). The M-value is further reduced when comparing the housekeeping gene expression between control and the two IFN subtype lupus patients. Furthermore, any combination of housekeeping genes is far below the V-value of 0.15 (**Figure 4.3**). Yields of RNA from neutrophils are in general very low, and due to small blood sample volumes from paediatric

patients and JSLE patients frequently having low neutrophil counts, RNA availability is very limited. As RNA content was limited in patient samples and housekeeping genes were very stable, it was decided that for neutrophils alone, only one housekeeping gene was measured. PBMCs analysed together with neutrophil cDNA varied stronger in housekeeping gene expression and it was therefore decided to use the three most stable genes which were UBC, ACTB and TOP1. As the same neutrophil RNA was used for PBMC-neutrophil and control-JSLE comparison we chose ACTB as the housekeeping gene for neutrophil comparison.

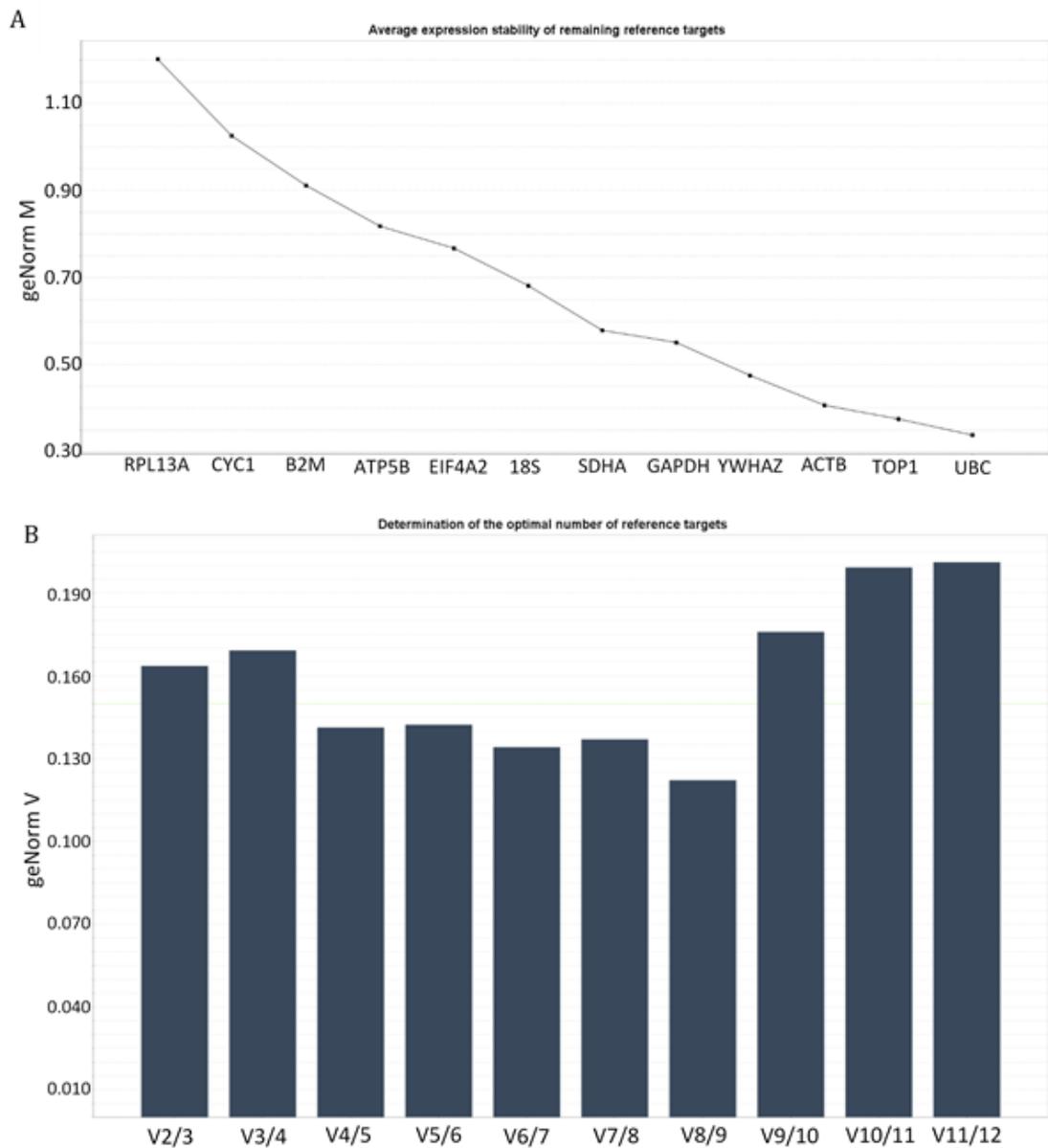


Figure 4.1: Reference target stability and optimal number of housekeeping genes for neutrophil and PBMC comparison. In total six cDNA samples of neutrophils and PBMCs were tested for their expression of 12 housekeeping genes. Stability of each gene is shown in (A) with the least stable gene on the left and the most stable gene on the right. GeNorm V in (B) shows how many genes are required to reach $V < 0.150$ by performing pairwise variation. Four genes (UBC, ACTB, TOP1 and GAPDH) are required for optimal normalization.

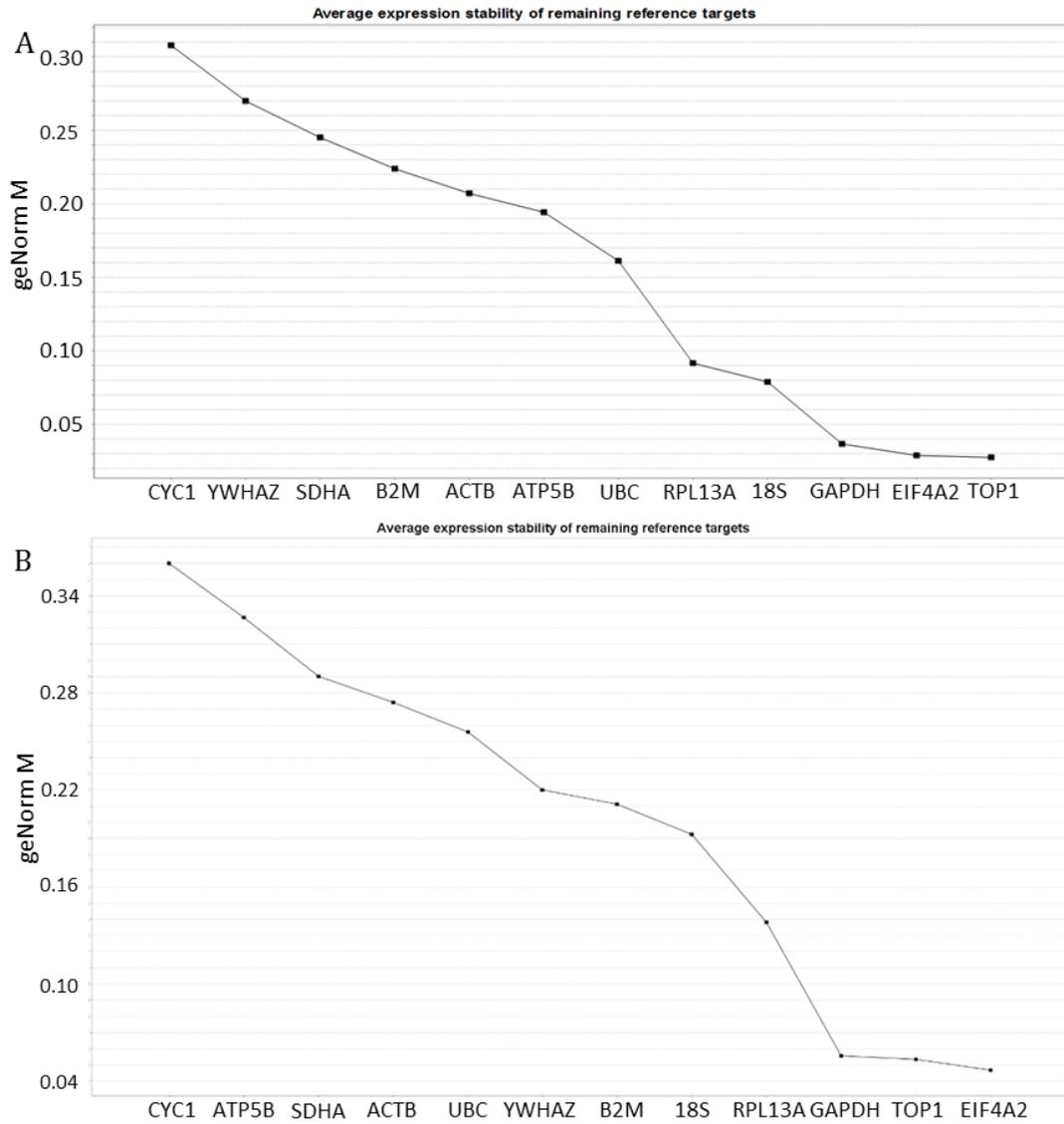


Figure 4.2: Housekeeping gene stability comparing control against JSLE patients and control against IFN high and low JSLE patients. In total six cDNA samples of neutrophils were tested for their expression of 12 housekeeping genes. Stability of each gene is shown as a value M with the least stable gene on the left and the most stable gene on the right. For both groups control and JSLE (A), and control with the JSLE IFN subtypes (B) all housekeeping genes show little variation as for each the value is below 0.5.

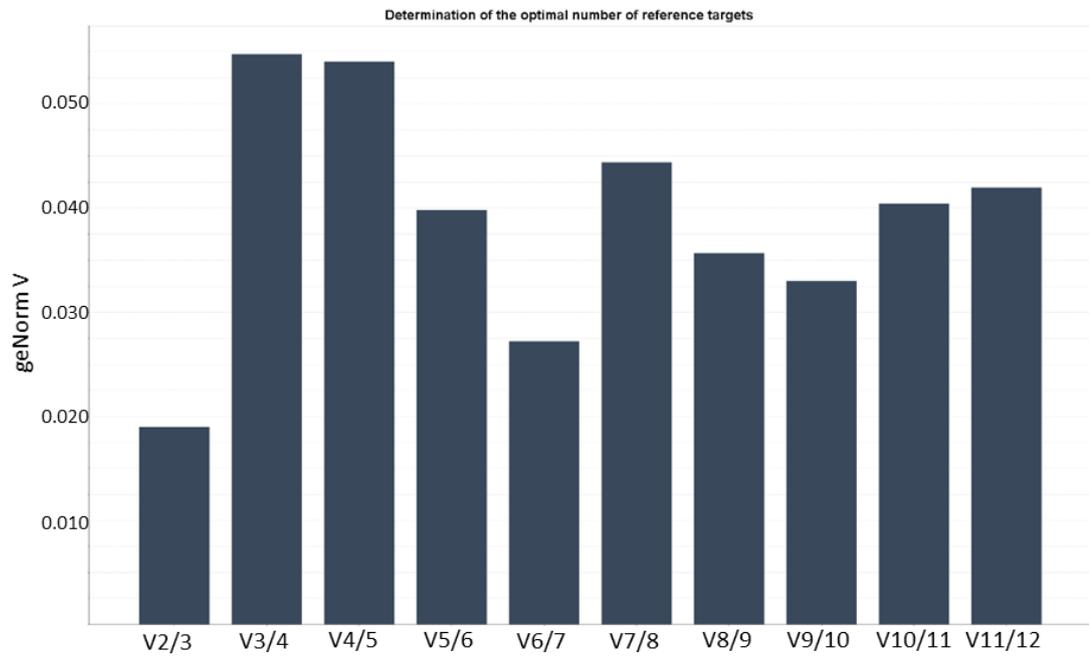


Figure 4.3: Determination of the optimal number of reference genes for control, IFN high JSLE and IFN low JSLE patients' neutrophils. Six cDNA samples of IFN subgroups and controls patients were used for analysis with qbasePLUS. The V-value has a cut-off at 0.15, but is very low for samples already with three housekeeping genes. From left to right an additional housekeeping gene is added and a new V-value calculated. Three housekeeping genes would consequently be the best option.

4.4.3 Determination of protein expression in serum using ELISA

Shed CD16b or released S100A8/S100A9 protein in supernatants and serum were measured using sandwich ELISA. CD16b was measured with an Aviva Systems Biology kit and release of S100A8/S100A9 was measured using DuoSet® ELISA DEVELOPMENT SYSTEMS from R&D systems. The protocol for the ELISA kits is described in Section 2.2.9.

For serum analysis patients were selected purely based on sample availability and knowledge of IFN high or low status as this could only be tested if RNA was retrievable. Used samples are summarised in **Table 4.2** for CD16b in **Table 4.3** for S100A8/S100A9.

	Control (N=8)	JSLE (N=11)	JSLE IFN high (N=6)	JSLE IFN low (N=5)
Age	11.4±3.4	15.3±5.7	16.2±6.9	14.3±4.3
Female	50%	78%	86%	75%
WB	100%	75%	75%	75%
Medication	none	HCQ (10/11) Mycophenolate (9/11) Prednisolone (4/11) Azathioprine (1/11) Methotrexate (1/11) no info (1/11)	HCQ (5/6) Mycophenolate (4/6) Prednisolone (1/6) Azathioprine (1/6) Methotrexate (1/6) no info (1/6)	HCQ (5/5) Mycophenolate (5/5) Prednisolone (3/5) Azathioprine (0/5) Methotrexate (0/5) no info (0/5)
Disease activity	ESR	17.5±18.2	23.2±22.7	10.4±8.1
	CRP	2.9±2.7	1.6±1.4	4.5±3.2
	C3	1.0±0.4	1.0±0.5	1.0±0.16
	C4	0.14±0.07	0.13±0.08	0.16±0.06
	dsDNA	15.9±23.4	31.3±29.6	3.6±8.0
	SLEDAI	6.4±5.0	4.0±2.2	8.8±6.1
	Renal BILAG	D (4/11) E (6/11) no info (1/11)	D (2/6) E (3/6) no info (1/6)	D (2/5) E (3/5) no info (0/5)

Table 4.2: Demographic and clinical data about patients whose serum was used for CD16b ELISA. Abbreviations: of White British ethnicity (%WB), Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Complement component 3 (C3), Complement component 4 (C4), anti-dsDNA titres (dsDNA), SLE Disease Activity Index (SLEDAI), British Isles Lupus Assessment Group (BILAG), Hydroxychloroquine (HCQ).

	Control (n=13)	JSLE (n=13)	JSLE IFN high (n=8)	JSLE IFN low (n=5)
Age	9.4±3.6	14.9±5.2	15.7±5.9	13.5±4.4
Female	62%	80%	83%	75%
WB	92%	70%	67%	75%
Medication	none	HCQ (12/13) Mycophenolate (11/13) Prednisolone (6/13) Azathioprine (1/13) Methotrexate (1/13) Aspirin (1/13) no info (1/13)	HCQ (7/8) Mycophenolate (6/8) Prednisolone (2/8) Azathioprine (1/8) Methotrexate (1/8) Aspirin (1/8) no info (1/8)	HCQ (5/5) Mycophenolate (5/5) Prednisolone (4/5) Azathioprine (0/5) Methotrexate (0/5) Aspirin (0/5) no info (0/5)
Disease activity	ESR	13.8±16.8	17.6±20.5	8.5±8.2
	CRP	3.15±2.4	2.3±1.7	4.4±2.8
	C3	1.0±0.5	0.9±0.6	1.1±0.2
	C4	0.18±0.12	0.19±0.15	0.17±0.06
	dsDNA	14.3±21.9	25±26.8	3.6±8.0
	SLEDAI	6.2±5.0	4.3±3.3	8.8±6.1
	Renal BILAG	D (5/13) E (7/13) no info (1/13)	D (3/8) E (4/8) no info (1/8)	D (2/5) E (3/5) no info (0/5)

Table 4.3: Demographic and clinical data about patients whose serum was used for S100A8/S100A9 ELISA. Abbreviations: White British ethnicity (WB), Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Complement component 3 (C3), Complement component 4 (C4), anti-dsDNA titres (dsDNA), SLE Disease Activity Index (SLEDAI), British Isles Lupus Assessment Group (BILAG), Hydroxychloroquine (HCQ).

Neutrophils defend the body against invading pathogens by uptake followed by digestion. This process had been shown to be dysregulated in lupus and was therefore investigated for this thesis – see Section 1.6.5 ([170]; [168]).

pHrodo™ BioParticles® Conjugates can represent different phagocytic targets like gram-positive (*S.aureus*), gram-negative bacteria (*E.coli*) or fungal particles (zymosan). Their fluorescent label changes brightness, dependent on the pH of the environment and they therefore emit light upon uptake into the phagosome which has a decreased pH.

Preliminary experiments were conducted in a POLARstar Omega plate reader (BMG Labtech) with a plate prepared as described in Section 2.2.7. Plates were then incubated for 85 min on the plate reader at 37°C and fluorescence was measured every 5 min. Under these conditions phagocytosis of neutrophils was observed of one control and one JSLE patient both with control and JSLE serum.

Surprisingly, JSLE neutrophils incubated with control serum showed higher uptake of particles at earlier time points (**Figure 4.4** *S.aureus* **A**, *E.coli* **B** and zymosan **C**) than in neutrophils of control patients.

Previously, phagocytosis has been observed at later time points where the effect of serum is more prominent on cells, but also has more effect on opsonization [170].

It was therefore decided to look at an early time point with a 20 min incubation. Bead uptake was analysed with a flow cytometer to ensure that fluorescence was not dependent on PBMCs, potentially present as contaminants in the sample, by gating the PMN population. Confocal microscopy was used to confirm the results visually.

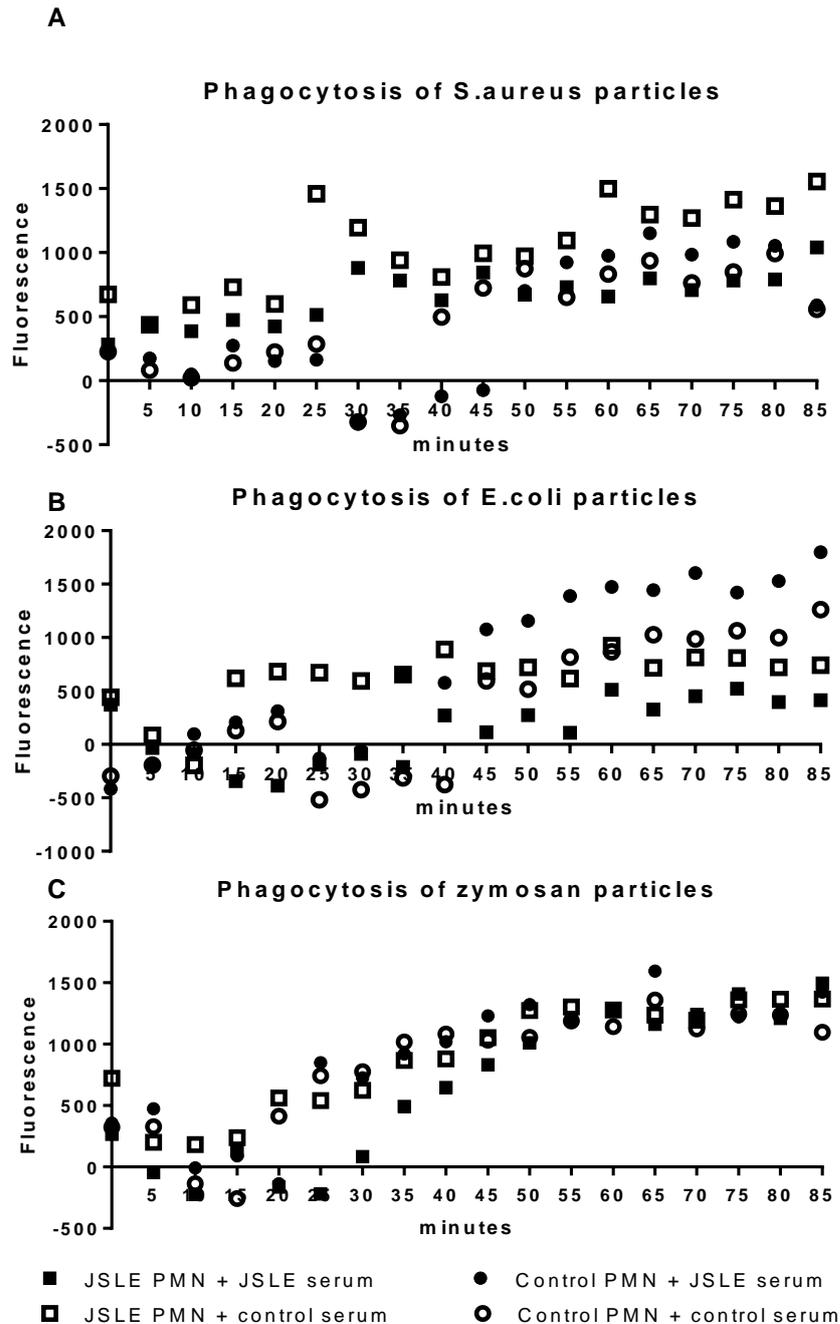


Figure 4.4: Phagocytosis assay of control and JSLE PMN taking up pHrodo-coated bioparticles. Neutrophils of one control (circle) and one JSLE patient (square) were tested for their ability to phagocytose pHrodo coated S.aureus (A), E.coli (B) and zymosan (C) bioparticles in the presence of control (unfilled) or JSLE serum (filled). Fluorescence was measured with a POLARstar Omega plate reader every 5 minutes for 85 minutes, fluorescence of particles alone and cells alone were subtracted, and results shown. In the presence of control serum, phagocytosis was higher in JSLE than control neutrophils, particularly at earlier time points. JSLE neutrophils with JSLE serum showed inhibited uptake for most time points.

4.4.4 Flow cytometry for analysis of phagocytosis assay and antibody staining for protein detection

Phagocytosis of pHrodo coated bioparticles was analysed with flow cytometry as described in methods in Section 2.2.7.

Neutrophils of JSLE patients (n=9) and control patients (n=8) were analysed for protein expression of TLR2, extra- and intracellular CD16b and S100A9 and patient information is summarised in **Table 4.3**. They were stained with antibodies as described in Section 2.2.6.

	<i>Control (n=7)</i>	<i>JSLE (n=9)</i>	<i>JSLE IFN high (n=6)</i>	<i>JSLE IFN low (n=3)</i>
Age	11.4±3.4	15.6±6.1	17.0±7.8	14.3±4.3
Female	50%	100%	100%	100%
WB	100%	83%	75%	100%
Medication	none	HCQ (8/9) Mycophenolate (7/9) Prednisolone (2/9) Azathioprine (1/9) Methotrexate (1/9) no info (1/9)	HCQ (5/6) Mycophenolate (4/6) Prednisolone (1/6) Azathioprine (1/6) Methotrexate (1/6) no info (1/6)	HCQ (3/3) Mycophenolate (3/3) Prednisolone (3/3) Azathioprine (0/3) Methotrexate (0/3) no info (0/3)
Disease activity	ESR	17.5±18.2	23.2±22.7	10.4±8.1
	CRP	2.9±2.7	1.6±1.4	4.5±3.2
	C3	1.0±0.4	1.0±0.5	1.0±0.16
	C4	0.14±0.07	0.13±0.08	0.16±0.06
	dsDNA	15.9±23.4	31.3±29.6	3.6±8.0
	SLEDAI	6.4±5.0	4.0±2.2	8.8±6.1
	Renal BILAG	D (3/9) E (5/9) no info (1/9)	D (2/6) E (3/6) no info (1/6)	D (1/3) E (2/3) no info (0/3)

Table 4.4: Demographic and clinical data about patients whose neutrophils was analysed for protein expression with flow cytometric analysis. Abbreviations: White British ethnicity (WB), Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Complement component 3 (C3), Complement component 4 (C4), anti-dsDNA titres (dsDNA), SLE Disease Activity Index (SLEDAI), British Isles Lupus Assessment Group (BILAG), Hydroxychloroquine (HCQ).

4.4.5 Analysis using confocal microscopy

As a visual representation, phagocytosis assays were also performed on cover slips and viewed under the confocal microscope. Assays were performed as described in Section 2.2.7.

4.4.6 Determination of overall CD16b expression or IGS by ranking

While the IGS consists of several genes, CD16b protein can be expressed intracellularly, on the cell surface or can be shed into serum. Therefore samples need to be investigated for each part of the IGS and CD16b individually, but to get an idea of the overall presence a scoring system was necessary in this thesis. As an example, scoring of CD16b is shown below in **Figure 4.5**. From left to right: All samples were ranked depending on their expression separately for cell surface CD16b and intracellular CD16b, measured with flow cytometry and results are shown in geometric mean fluorescence. They were also ranked for CD16b concentrations shed into serum which was measured with ELISA. The average rank was then calculated by:

$$\frac{\text{rank CD16b cell surface} + \text{rank CD16b intracellular} + \text{rank CD16b serum}}{3}$$

Cell surface CD16b			Intracellular CD16b			Serum CD16b		
	geometric mean	Rank 1-16		geometric mean	Rank 1-16		concentration	Rank 1-16
Control1	3860	15		24.1	2		2746.72	11
Control2	1605	3		1067	12		3261.6	12
Control3	2646	8		809	6		3465.44	14
Control4	1539	2		852	8		410.88	1
Control5	2941	10		23.5	1		978.4	3
Control6	2475	7	+	918	9	+	2149.6	7
Control7	2421	6		634	4		2393.28	9
JSLE1	2411	5		650	5		2295.36	8
JSLE2	1413	1		943	10		1481.28	5
JSLE3	2345	4		1202	13		3908.16	16
JSLE4	3261	14		1223	14		3536.32	15
JSLE5	2955	11		966	11		3438.08	13
JSLE6	2889	9		1303	15		984.96	4
JSLE7	3102	12		210	3		2120.32	6
JSLE8	4116	16		1387	16		2443.2	10
JSLE9	3216	13		848	7		507.36	2

	Sum of all	Sum divided by three
Control1	28	9.33
Control2	27	9.00
Control3	28	9.33
Control4	11	3.67
Control5	14	4.67
Control6	23	7.67
Control7	19	6.33
JSLE1	18	6.00
JSLE2	16	5.33
JSLE3	33	11.00
JSLE4	43	14.33
JSLE5	35	11.67
JSLE6	28	9.33
JSLE7	21	7.00
JSLE8	42	14.00
JSLE9	22	7.33

= Sum/3

Figure 4.5: Example for the used scoring system with CD16b as an example. At the top, from left to right, are samples ranked for CD16b cell surface and intracellular expression and shed CD16b. Expression of cell surface CD16b and intracellular CD16b were measured with flow cytometry and results are shown in geometric mean fluorescence. All sample were also ranked for CD16b concentrations shed into serum which was measured with ELISA. The average rank is then calculated as the sum of all three ranks for each sample (e.g. JSLE1 CD16b cell surface+JSLE1 CD16b intracellular+JSLE1 CD16b serum) divided by three.

4.5 Results

Transcriptomics analysis of PMN by our group had revealed that within our JSLE cohort there are patients who can be distinguished by an IGS. Further analysis showed that there are differences in several other genes between patients classified as IFN high and low depending on their signature. The NCBI gene library (<https://www.ncbi.nlm.nih.gov/gene/?term=phagocytosis>) and NCBI PubMed library (<https://www.ncbi.nlm.nih.gov/pubmed/>) was used to search for functions of genes with significant difference. Two main functions were observed, which were modification of DNA and phagocytosis related genes (PRGs). IFN induced, DNA related and phagocytosis related genes were investigated and validated with real-time PCR.

4.5.1 The IFN-induced gene signature in JSLE patients

4.5.1.1 Presence of IGS in neutrophils and PBMC

IFN signature was described in whole blood of JSLE patients [57], but was also found in different subpopulations of cells in RA [56]. The latter described neutrophils to be especially sensitive to IFN signalling. A validation of IGS expression in neutrophils compared to PBMC's was necessary within our paediatric cohort as the previous study was based on adult RA patients.

As **Figure 4.6** shows, OAS2, IFI44L, LY6E and IFI6 were expressed more in the neutrophil (PMN) population compared to the PBMC fraction. OAS2, IFI44L and LY6E was expressed less in the PBMC fraction in all patients. IFI6 mRNA, however, was higher in one patient's PBMCs compared to the respective neutrophils. These results were sufficient to decide that neutrophils were the more sensitive cell type to investigate the influence of the IGS.

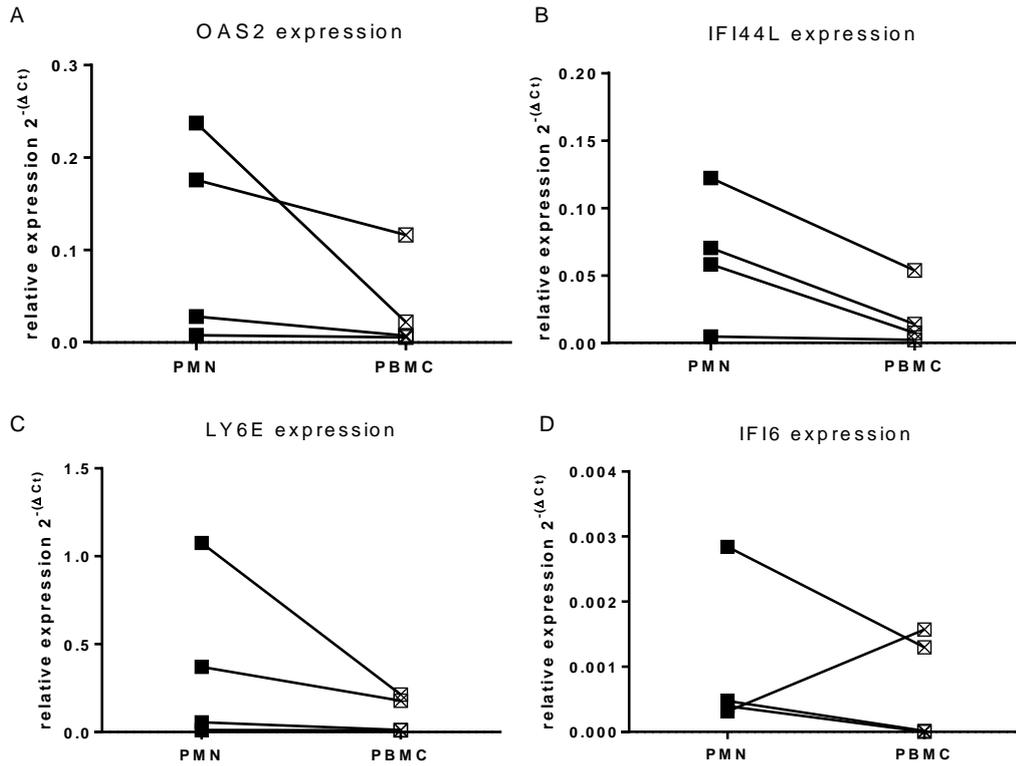


Figure 4.6: Expression of IFN-induced genes in neutrophils and PBMCs of JSLE patients. Neutrophil (PMN) and PBMC mRNA of four patients was measured for expression of OAS2 (A), IFI44L (B), LY6E (C) and IFI6 (D) which can be induced by IFN. Real-time PCR using SYBR green showed higher expression of IFN-induced genes for neutrophils in all genes and all samples except for one sample which had higher levels of IFI6 in the PBMC fraction. Relative expression was calculated taking the deltaCt-value of the gene of interest and the geometric mean of the three house keeping genes UBC, ACTB and TOP1. Statistical analysis was not applied to these data as only n=4 were compared.

4.5.1.2 Changes of IFN-induced genes over time

Even though the IGS has been described before ([56],[57]), there are no data comparing how the signature changes over time in a JSLE cohort. The cohort of patients in this study have recurrent hospital visits, so in addition to their study number they also get an episode number unique to each hospital visit. This enables us to compare the changes in IFN-induced genes longitudinally. We monitored five patients (**Figure 4.7**) of which patients L43 and L62 were considered IFN low when they were initially classified by transcriptomics data. RNA of three episodes for L43 and two episodes for all other samples was analysed. Disease activity was not consistent over time and especially worth mentioning, L43 and L62 had both high and low disease activity over the time course. The later L62 time point corresponded to the patient having a SLEDAI of 0 whereas at the earlier time point the patient had a SLEDAI of 16. Additionally, to our knowledge none of the patients had infections. The expression of IFN-induced genes had changed strongly when comparing the different episodes for the IFN high patients L54, L68 and L73. For L54 at the later episode even a decrease was observed, but for all patients the signature remained a high one throughout. While L43 of the IFN low patients did not show any changes in the IGS, L62 developed higher IFI6 expression.

This raises the question if in fact, all of these four genes are actually good indicators to decide between an IFN high and IFN low signature.

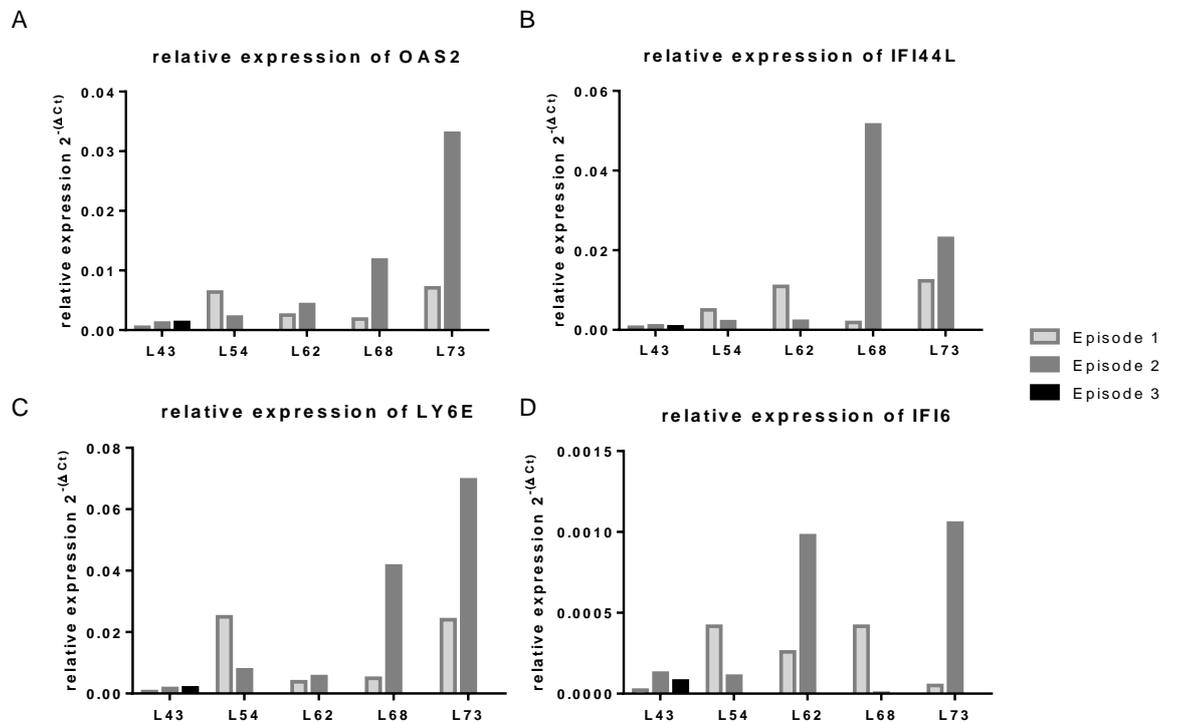


Figure 4.7: Relative expression of IFN-induced genes in different episodes of five patients. mRNA expression of OAS2 (A), IFI44L (B), LY6E (C) and IFI6 (D) in neutrophils (n=5 JSLE patients) was measured using SYBR green qRT-PCR and results were normalized to ACTB as a house keeping gene. For L43 three time points were tested whereas for all other patients only two episodes were available. L43 remains with very low IFN-induced gene expression. L62 previously also classified as an IFN low patient shows increase in mainly IFI6. L54, L68 and L73 were classified as IFN high patients and remained with this signature for the analysed time-point. L54 showed a decrease in all genes, but still remained an IFN high patient.

4.5.1.3 Evaluation of IFN low and IFN high patients

Having confirmed that neutrophils are an appropriate and important cell type in which to study the IFN gene signature, we wanted to confirm expression of the selected genes OAS2, IFI44L, LY6E and IFI6 in additional patients from the JSLE cohort. Through this method, patients could be classified as being IFN high or IFN low.

We therefore compared IFN-induced genes between paediatric control patients and an additional number of JSLE patients in which samples were available. Notably, there was a significant increase of all selected IFN-induced genes in 13 JSLE patients compared to 13 controls (**Figure 4.8A-D**) with the most significant difference in OAS2 expression ($p=0.0006$). This was followed by IFI44L ($p=0.0051$) and LY6E ($p=0.003$) with the smallest difference in IFI6 ($p=0.0499$). Two lupus samples fell within the 95% confidence interval of the control group (cut-off indicated as a dashed line) for all four genes, but for IFI6 and IFI44L there were additional patients within the control range. We therefore used all four genes as a panel to evaluate the IGS of the patients. Samples were ranked for each gene and then the average of ranks was calculated for each sample with the result shown in **Figure 4.8 (E)**. Significantly higher ranks were observed for JSLE patients ($p=0.0027$). Four JSLE patients were found to be lower than the minimum of the 95% confidence interval and were also below the maximum of the 95% confidence interval of control patients. The patients were therefore classified as overall “IFN low” patients.

Even though it is classified as one “IFN-induced gene signature”, genes may be influenced by different or at least additional signals. Gene expression of all four genes in different episodes of five JSLE patients was measured with qRT-PCR. Comparing the expression of the genes in pairs can reveal correlation. High correlation would indicate that they might be influenced by the same stimulus or have an impact on each other. As shown in **Figure 4.9**, we found significant correlation (Spearman correlation test) between OAS2 and IFI44L (**A**, $r=0.955$, $p=2.8 \times 10^{-5}$), OAS2 and LY6E (**B**, $r=0.945$, $p=4.9 \times 10^{-5}$) and IFI44L and LY6E (**C**, $r=0.86$, $p=0.001$). None of the genes showed significant correlation with IFI6 (with IFI44L $r=0.132$ $p=0.698$, OAS2 $r=0.246$ $p=0.463$, LY6E $r=0.246$ $p=0.463$).

The chosen IGS, comprising OAS2, IFI44L, LY6E and IFI6, appears to cover different stimuli. IFI6 was therefore excluded from the classification criteria for an “IFN signature” in this work.

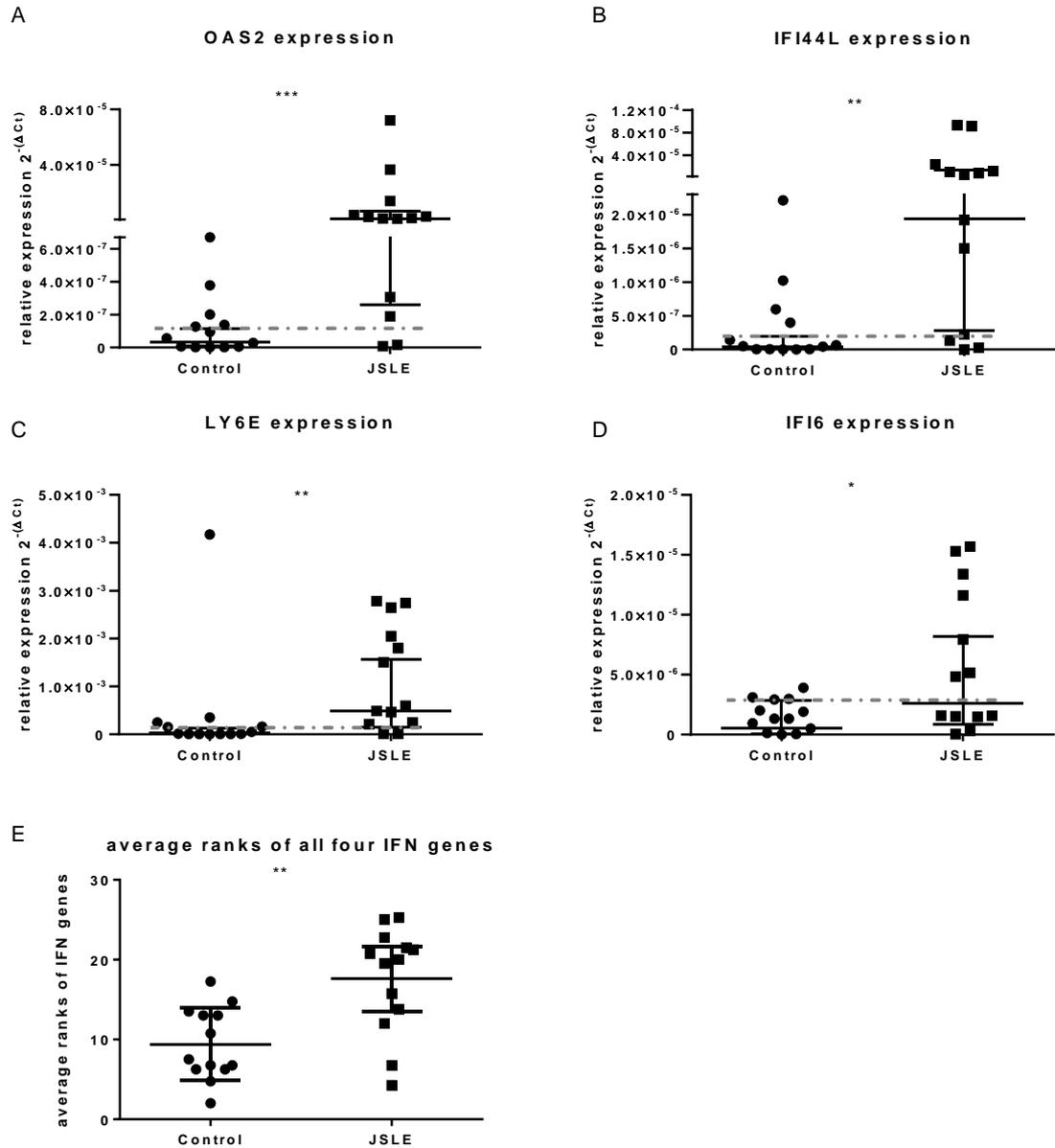


Figure 4.8: Increased expression of IFN induced genes in JSLE patients compared to healthy controls and separation of patients into IFN low and IFN high patients. mRNA expression of OAS2 (A), IFI44L (B), LY6E (C) and IFI6 (D) (n=13 each JSLE and control patients) was measured using SYBR green qRT-PCR (Agilent Lifetechnologies) and results were normalized to ACTB as a house keeping gene. Results are presented as individual samples with circles (control) and squares (JSLE), with the geometric mean and 95% confidence interval (CI). The dashed line indicates the maximum of the control CI. Samples were then ranked for each gene and plotted as the average rank for all genes to determine if patients were IFN high or IFN low (E). Cut-off was the minimum of the JSLE 95% CI. *p<0.05 ** p<0.01 *** p<0.001 Mann-Whitney test

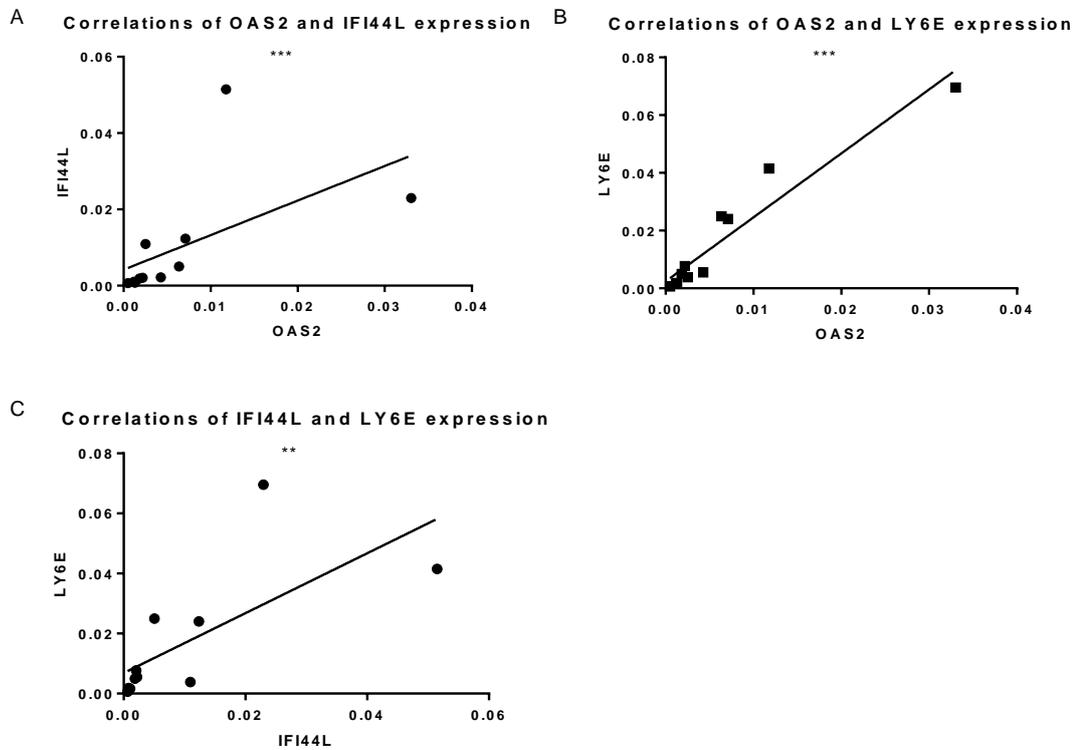


Figure 4.9: Significant correlation of IFN-induced genes OAS2, IFI44L and LY6E. mRNA expression of five JSLE patients was measured at two or three different episodes allowing a comparison of n=11 using qRT-PCR with SYBR green. Values are presented as relative expression, calculated by normalizing the gene of interest to ACTB. Correlation was observed between OAS2 and IFI44L (A), OAS2 and LY6E (B) and IFI44L and LY6E (C) (regression $r=0.955$, $r=0.945$ and $r=0.86$ respectively). ** $p<0.01$ *** $p<0.001$ Spearman correlation

4.5.2 Expression of genes of DNA-related proteins in JSLE

The IGS has been described in several papers [56], [57], but patients were not further characterized. Transcriptomics data from our group showed more differences between IFN high and IFN low patients. Several changes, for example, indicated genes of DNA-related proteins to be altered. Gene expression of these proteins between not only control and JSLE patients but also the IFN subgroups was therefore compared.

Some of the proteins resulting from these genes can modify histones to alter transcription. One example is the SET domain containing lysine methyltransferase 7 (SETD7) which causes methylation of lysine-4 on histone 3 leading to activation of transcription [239]. It can also methylate non-histone proteins and is, for example, involved in ROS signalling [240]. Another example is SAM domain, SH3 domain and nuclear localization signals 1 (SAMSN1) which interacts with Sin3A Associated Protein 30 (SAP30) and histone deacetylase 1 increasing deacetylase activity and thereby inhibiting transcription [241].

Other genes are important for DNA repair such as TREX1 for proofreading [242] or X-Ray Repair Cross Complementing 4 (XRCC4), Snf2-Related CBP Activator Protein (SRCAP) and Breast Cancer 2 (BRCA2) for double strand break repair [243], [244] [245].

As seen in **Figure 4.10**, there were no significant differences between control and JSLE or the IFN subtypes in SAMSN1, SAP30, SETD7, XRCC4 and SRCAP mRNA expression. A significant increase of BRCA2 was found for JSLE patients compared to healthy paediatric controls patients. This difference was very much influenced by the IFN high group. While the IFN low group was not different to the controls, the IFN high group showed increased ($p=0.06$) BRCA2 expression. Similar results were found for TREX1 which was significantly higher in JSLE patients and in IFN low patients was similar to the control group whereas IFN high patients were responsible for the observed increase ($p=0.003$). Overall, there were no strong differences between JSLE and control patients or after separation into IFN high and low patients.

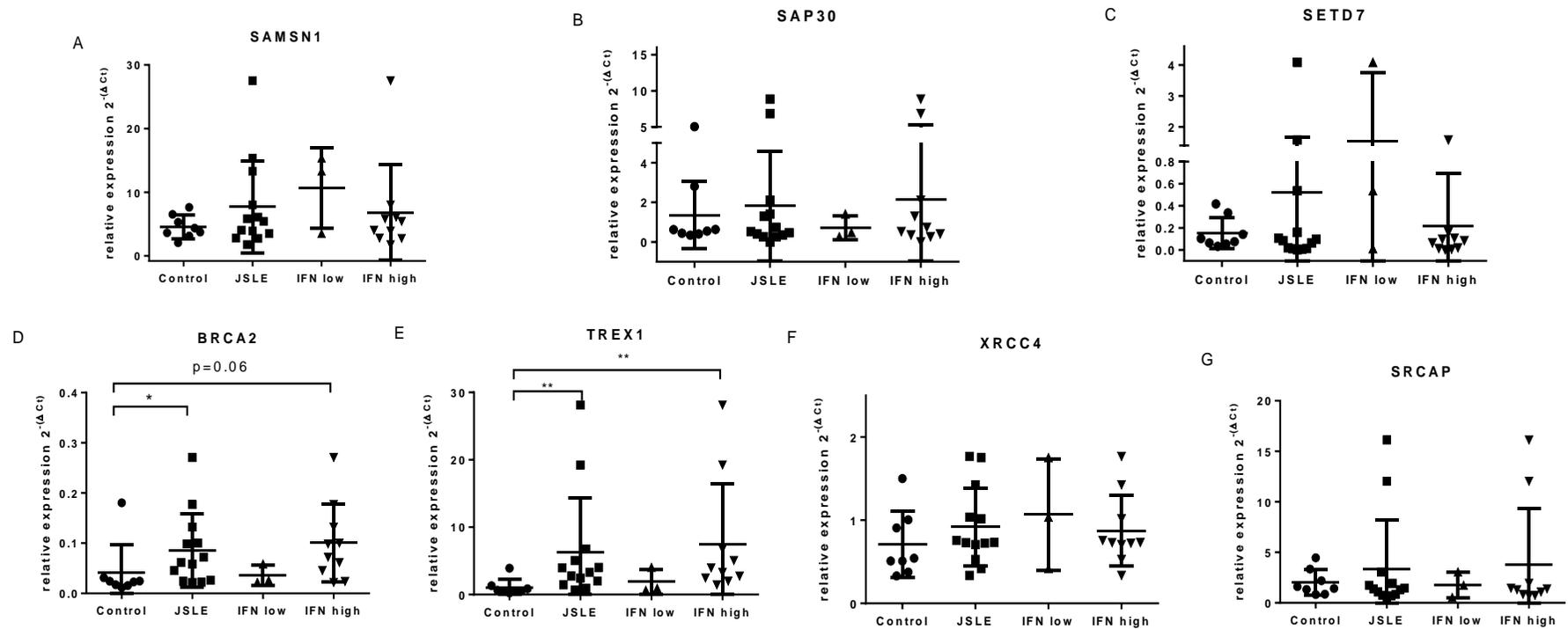


Figure 4.10: mRNA expression of DNA related proteins in JSLE, IFN stratified JSLE patients and healthy paediatric control patients. mRNA expression of DNA related proteins namely SAMSN1 (A), SAP30 (B), SETD7 (C), BRCA2 (D), TREX1 (E), XRCC4 (F) and SRCAP (G) was measured using SYBR Green real-time PCR. All results were normalized to TOP1 and EIF4A2 as suggested by GeNorm analysis and shown as relative expression. Only for BRCA2 and TREX1 significant increase was observed for the JSLE cohort (n=13). JSLE IFN high (n=10) patients caused these changes with a non-significant increase of BRCA2 (p=0.06) and a significant increase in TREX1. IFN low patients (n=3) showed expression like the control cohort (n=8). Mann-Whitney test for JSLE-Control comparison, Kruskal-Wallis test for IFN low, IFN high, Control comparison *p<0.05 **p<0.01

4.5.3 Phagocytosis related genes are differentially expressed in JSLE

The transcriptomic analysis from our group also suggested PRG to be differentially expressed.

Beginning with binding of the pathogen, phagocytosis involves several separate processes which are equally important for an efficient clearance of infection. These steps include targeting material with proteins such as AnxA3 [202], recognition via receptors like TLR2, FcγRIIb, CR3, and Dectin-1 ([180], [195], [236], [171]) processing of the phagosome via CamK1D [207] and stimulation of phagocytosis via S100A9 [198]. If any of these genes are dysregulated, neutrophils could become incapable of phagocytosis and instead produce NETs.

In order to analyse relevant phagocytic genes, the expression of TLR2, FcγRIIb, CR3, AnxA3, Dectin-1, CamK1D and S100A9 was measured. For this purpose, RNA from neutrophils of control and JSLE patients was extracted with TRizol, cleaned up and transcribed into cDNA as described in Section 2.2.5 Gene expression was then measured with qRT-PCR using SYBR Green I, a dye that intercalates into double-stranded DNA (dsDNA) and only then emits fluorescence [246]. Amplification of the target gene results in increased quantity of dsDNA and therefore a stronger fluorescent signal.

As **Figure 4.11** shows, there is a trend in neutrophils of JSLE patients to express more FcγRIIb, CR3 and AnxA3 compared to healthy paediatric control patients. TLR2 was 1.84-fold ($p=0.003$) and S100A9 1.56-fold higher ($p=0.012$) in JSLE patients. While there was no difference for Dectin-1 between the two groups a trend for decrease in CamK1D ($p=0.17$) was detected.

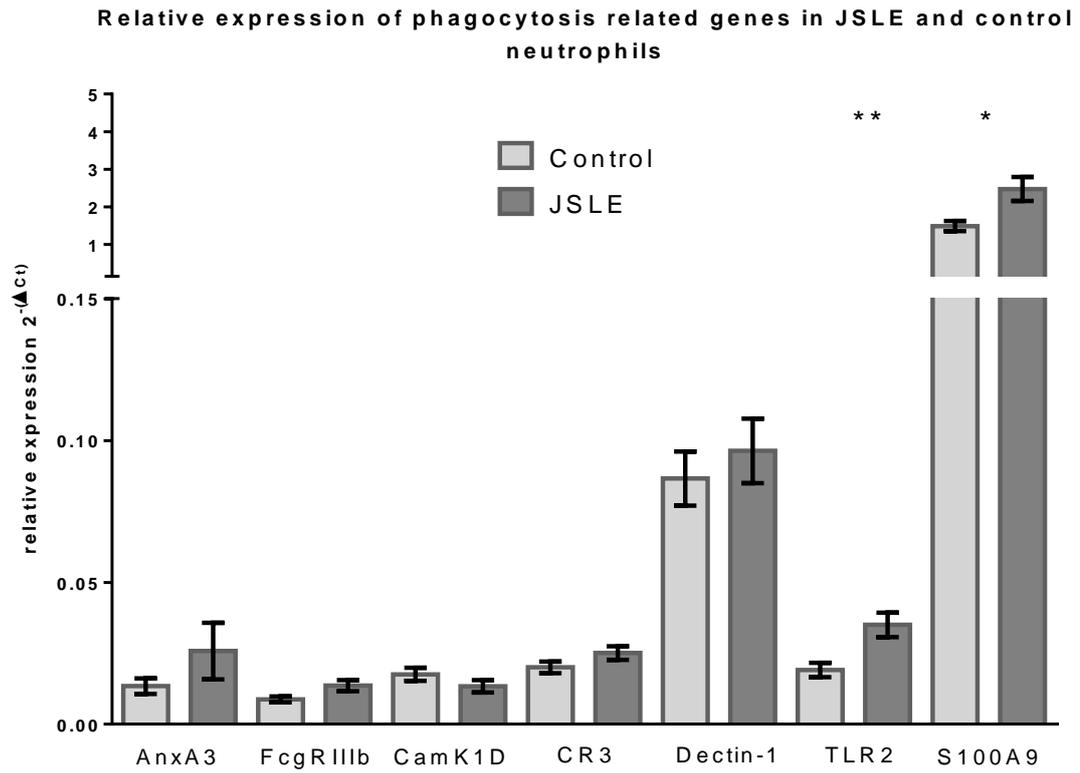


Figure 4.11: The majority of phagocytic genes is increased in JSLE patients compared to paediatric healthy control patients. mRNA expression of phagocytic genes in JSLE and control neutrophils was measured using SYBR Green qRT-PCR (Agilent Lifetechnologies). Each gene was normalized to ACTB and is presented as mean of the relative expression with SEM for each group. Results represent for both groups 13 patients. * $p < 0.05$ ** $p < 0.01$, performing a Mann-Whitney test

OAS2, IFI44L and LY6E RNA expression was tested for all samples to distinguish between IFN low and IFN high patients as described in Section 4.5.1 and PRGs compared between healthy paediatric controls patients, JSLE IFN high and JSLE IFN low patients **Figure 4.12**. IFN low patients did not show significant difference between any of the genes and only AnxA3, TLR2 and S100A9 showed a trend for increased expression. However, patients with an IFN high signature were significantly higher for TLR2 ($p=0.022$) with a 1.90-fold, S100A9 ($p=0.047$) with a 1.66-fold and Fc γ RIIb ($p=0.012$) with a 1.88-fold change compared to the healthy paediatric control patient group. Additionally, in IFN high patients, Fc γ RIIb was significantly upregulated compared to IFN low patients ($p=0.017$). A non-significant reduction for CamK1D (0.67-fold decrease) was observed in the IFN high compared to the control group. IFN high patients are therefore differently regulated than controls, whereas IFN low patients resemble the control group.

Three of the PRGs were noted to be differentially expressed compared to two DNA related genes and metabolomics analysis as described in Chapter 3 suggested importance of phagocytosis in JSLE. The PGS was therefore chosen for further investigation.

Relative expression of phagocytosis related genes in control and IFN subgroups

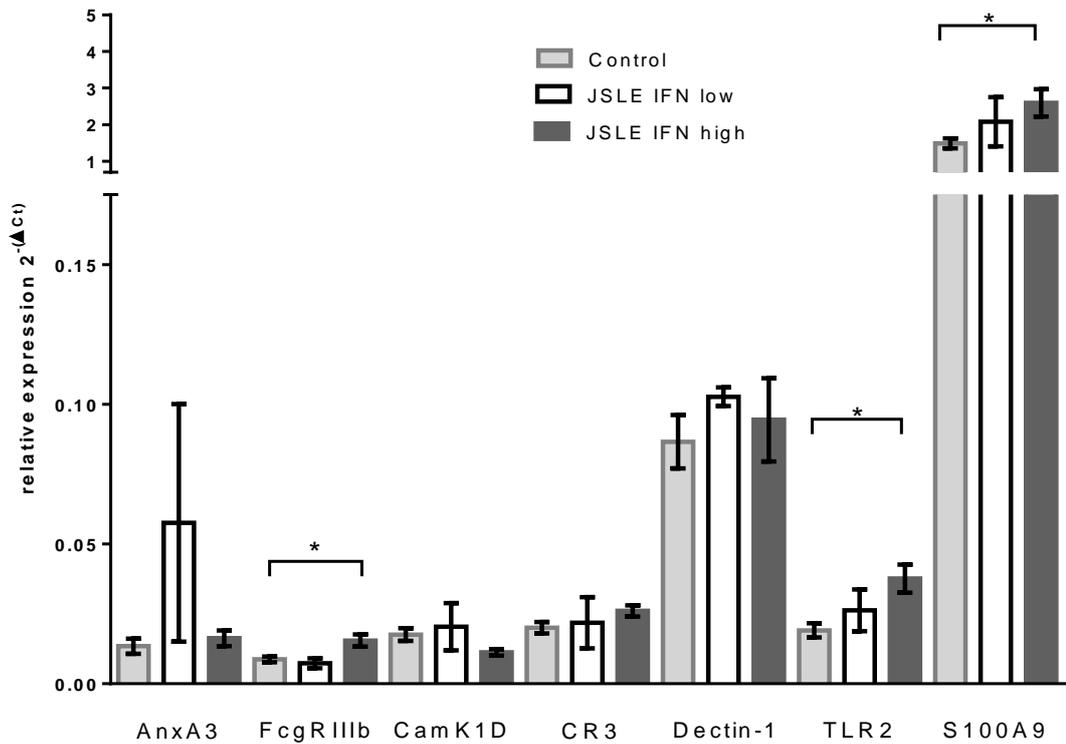


Figure 4.12: IFN high patients differ more from control patients in their phagocytic gene profile than IFN low patients. mRNA expression of phagocytic genes in JSLE and control neutrophils was measured using SYBR Green qRT-PCR (Agilent Lifetechnologies). Each gene was normalized to ACTB and is presented as mean of the relative expression with SEM for each group. Results represent 13 control, three IFN low and 10 IFN high patients. * $p < 0.05$, performing a Kruskal-Wallis test

4.5.4 Protein expression

In Section 4.5.3, it was noted that phagocytosis related genes are differentially expressed in JSLE and that mRNA of TLR2 and S100A9 were differently expressed in the neutrophils of healthy paediatric control patients and JSLE patients. When splitting the patients into IFN subgroups these differences were mainly due to high expression in IFN high patients whereas IFN low patients appeared to be more similar to healthy control patients. After this separation it also became clear that FcγRIIIb is high in IFN high patients, but not in IFN lows. Changes observed in mRNA levels do not give certainty about translation into protein and therefore the protein expression of TLR2, S100A9 and CD16b, the protein of FcγRIIIb, was measured. TLR2 was analysed for cell surface expression, S100A9 for intracellular and CD16b for both cell surface and intracellular protein expression. As S100A9 can be secreted and is most commonly found in complex with S100A8. For quantification of the bioactive form, the presence of the S100A8/S100A9 heterodimer was measured in serum using ELISA. CD16b can be shed from the cell surface and was therefore also quantified with ELISA.

Protein expression of TLR2 was significantly higher in the JSLE cohort compared to healthy controls ($p=0.02$, **Figure 4.13(A)**) The IFN low group was primarily responsible for the TLR2 increase in the JSLE cohort being significantly higher ($p=0.01$) compared to the control group, whereas the IFN high group was only increased non-significantly. Intracellular S100A9 (**B**) was significantly increased between the control and JSLE group with the lupus cohort expressing more protein. The S100A8/S100A9 heterodimer (**C**) was found to be non-significantly increased ($p=0.11$) for the JSLE cohort which again was mainly caused by the IFN low group.

The protein expression of CD16b (**Figure 4.14**) needs to be measured intra- (**A**), extracellularly (**B**) as well as in serum (**C**) to obtain a full picture of the protein expression. For each assay samples were ranked and then the average rank for each sample was calculated (**D**) as described in 4.4.6.

CD16b did not show a significant difference between JSLE and healthy paediatric control patients (p=0.72 extracellular, p=0.07 intracellular, p=0.81 serum and p=0.21 scored CD16b expression). No significant difference was observed between the IFN groups and the control group, potentially due to the low numbers of IFN low patients (p=0.71 extracellular, p=0.12 intracellular, p=0.81 serum and p=0.36 scored CD16b expression). Nevertheless, these data show a trend for lower CD16b expression in IFN low patients compared to IFN high patients for intracellular CD16b as it was suggested in the qPCR data.

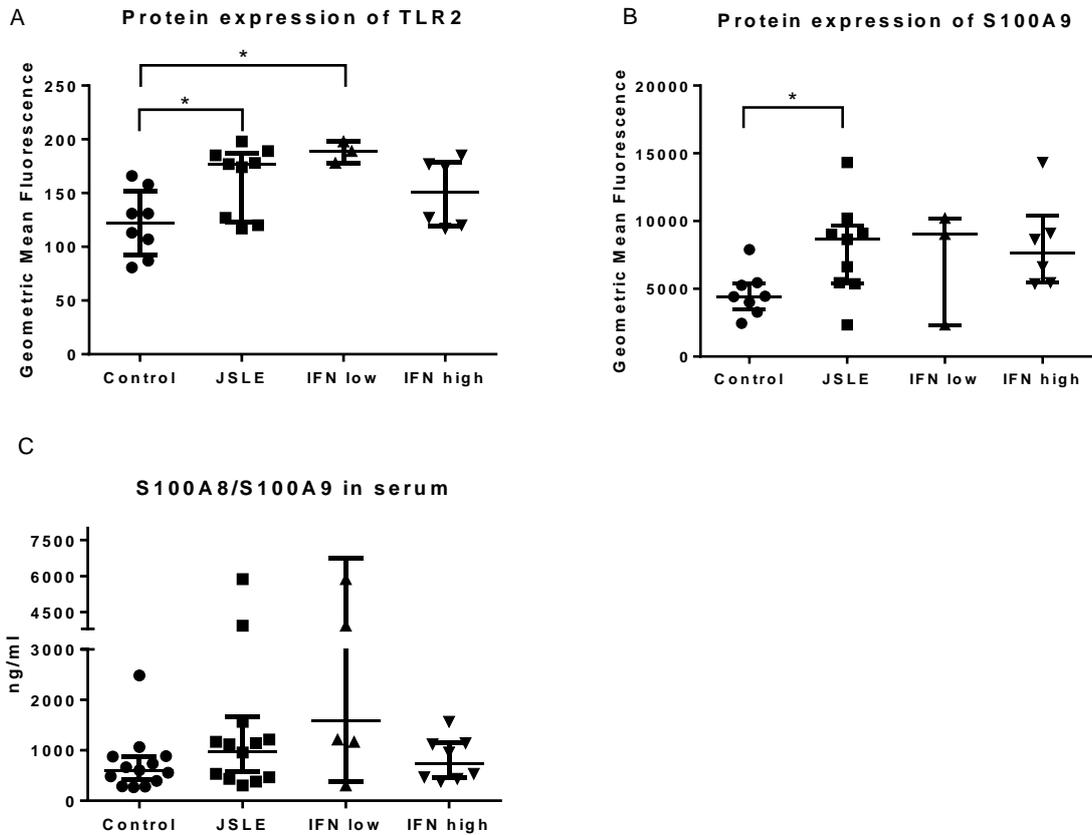


Figure 4.13: Protein expression of TLR2, S100A9 in JSLE and control neutrophils and released S100A8/S100A9 in serum. Protein expression of TLR2 (A) and S100A9 (B) was measured using flow cytometry in healthy controls (n=8), and JSLE patients (n=9) of which three were IFN low and six were IFN high patients. The presence of the heterodimer S100A8/S100A9 in patients' sera was measured with ELISA (C) for n=13 healthy controls and n=13 JSLE patients of which n=5 were IFN low and n=8 were classified IFN high. Significant higher protein expression was found in JSLE patients compared to healthy controls for both TLR2 and S100A9. Additionally, TLR2 expression was significantly higher in the IFN low group compared to healthy controls. No significant difference was observed for S100A8/S100A9 in serum. Mann-Whitney test for JSLE-Control comparison, Kruskal-Wallis test for IFN low, IFN high, Control comparison *p<0.05

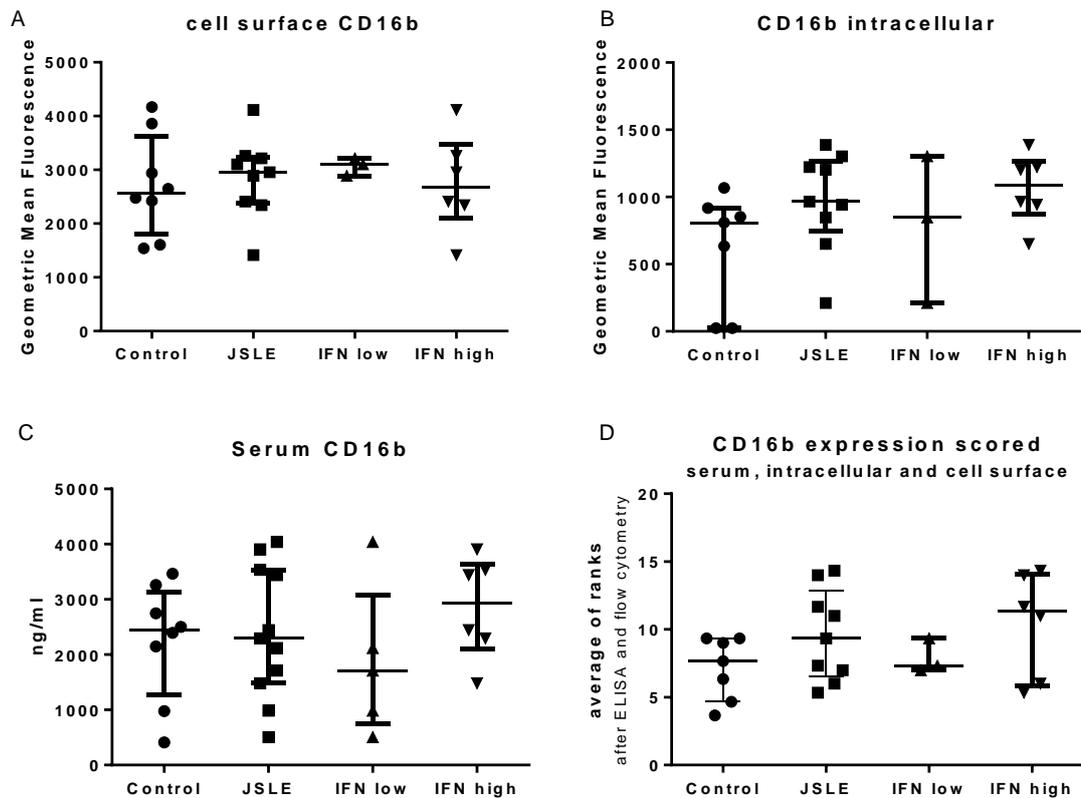


Figure 4.14: CD16b protein expression in healthy paediatric control patients and JSLE patients, as well as their IFN subtypes. Cell surface CD16b (A) expression was measured with flow cytometry for n=8 and intracellular CD16b (B) for n=7 healthy paediatric control patients. The same measurement was undertaken for n=9 JSLE patients of which n=3 were IFN low and n=6 were IFN high. Serum CD16b levels (C) were measured using ELISA in serum of n=8 healthy controls and n=11 JSLE patients of which n=5 were IFN low and n=6 were IFN high subtype. For the samples where data was available for all three assays, samples were ranked from 1 to 16 (total number of samples) for each assay and then the average of the ranks was calculated for each sample as displayed in (D). Mann-Whitney test for was performed for JSLE-Control comparison, Kruskal-Wallis test for IFN low, IFN high, Control comparison p<0.05 was considered significant

4.5.5 Phagocytosis of pathogens

To fully assess whether the mRNA and protein expression of these factors may have any effect on the functions of cells or if other proteins may compensate for this dysregulation, the phagocytic activity of neutrophils was investigated.

Neutrophils have different receptors for specific types of microorganisms. These tests need to cover a range of pathogens as a faulty or dysregulated receptor targeting a certain pathogen may not affect uptake of other microbes. Most studies restricted the comparison of lupus and control patients' ability to phagocytose on one to two microorganisms ([170], [4]). Selected targets should include pathogens both from bacterial, with the subgroups of gram positive and gram negative strains, as well as fungal origin in order to ensure that different recognition and uptake mechanisms are tested.

The efficiency of phagocytosis was measured with pHrodo coated *S.aureus*, *E.coli* and zymosan (Molecular Probes) particles. These particles emit fluorescence once taken up into the phagosome due to the more acidic environment. This effect can be detected with flow cytometry as cells which have not engulfed any bacteria are non-fluorescent and can be distinguished from pHrodo positive cells. To confirm the results observed with flow cytometry, cells were seeded onto coverslips before incubation with particles and were visualised with confocal microscopy.

To minimise the effects of a potential complement deficiency in the serum and observe only the neutrophil intrinsic ability, phagocytosis assays were performed without pre-opsonisation and only for 20 minutes at 37°C. Furthermore, the cells were incubated in parallel with JSLE and control serum or without serum.

Phagocytosis with little effect of serum would suggest that mainly TLR2 and S100A9 are involved in this process which are a receptor for pathogen recognition and a phagocytosis stimulator, rather than CD16b a receptor mainly responsible for immune complexes. No difference was observed comparing the IFN subgroups for TLR2 and S100A9 and we therefore did not split the patients into IFN low and IFN high for this assay.

4.5.5.1 Phagocytosis of *S.aureus* by neutrophils

After 20 min there was no difference of uptake observed between any of the incubations and about 100% of cells have phagocytosed (**Figure 4.15 (A)**). Confocal pictures suggested a slightly higher uptake for healthy paediatric control patients (**C+D**) compared to PMN of JSLE patients (**E+F**). Particles need to settle first to reach the neutrophils which were left to adhere for 45 min before stimulation with particles. This suggests that at an even earlier time point may be needed to observe a difference for flow cytometry.

For this reason, we also looked at the 20 min incubation at 37°C without any serum as this should slow down phagocytosis and might give a suggestion if there is a difference between the neutrophils of JSLE and control patients (**Figure 4.16**). Indeed, JSLE neutrophils showed an enhanced (non-significant ($p=0.18$)) uptake of *S.aureus* particles without serum.

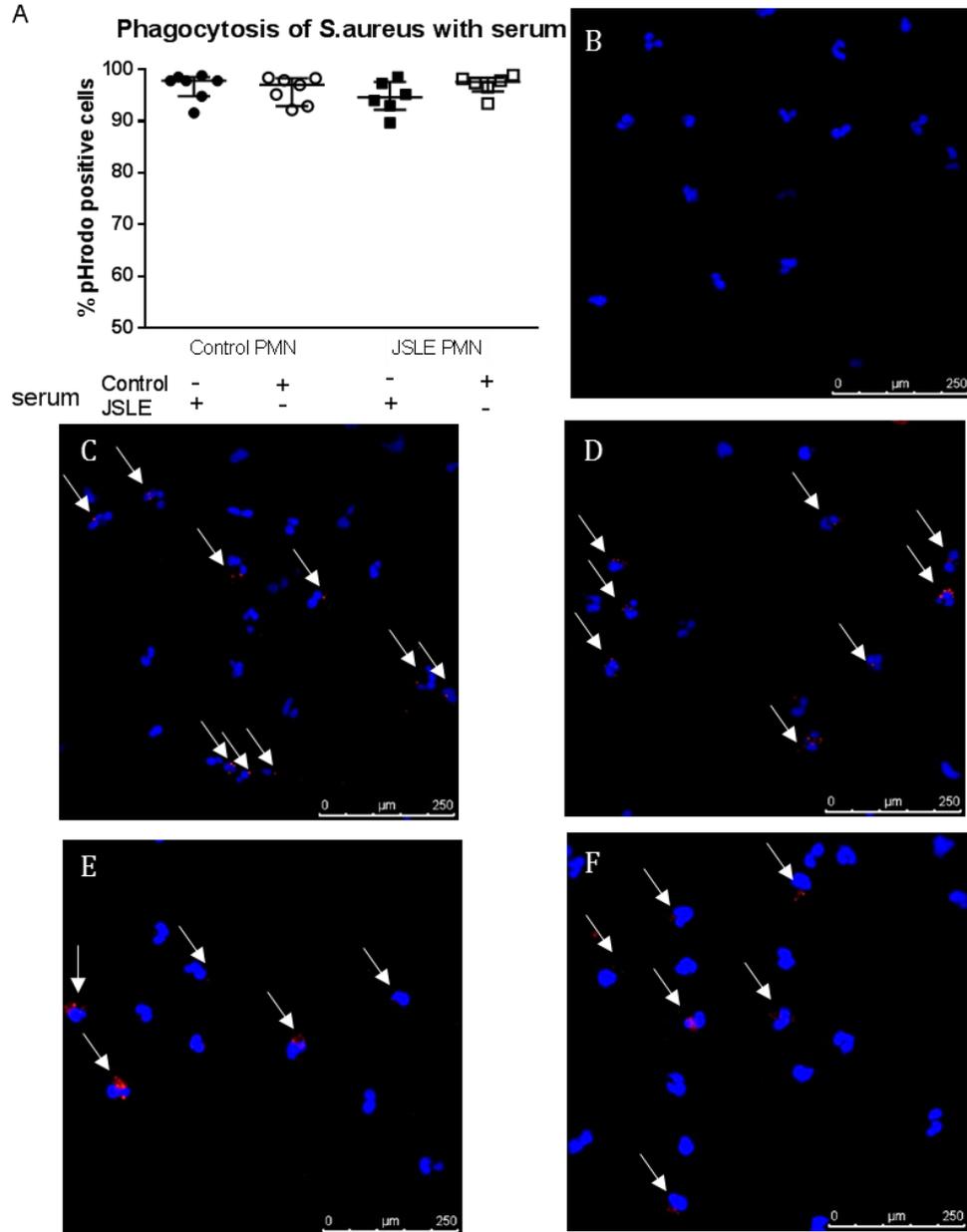


Figure 4.15: JSLE neutrophils can phagocytose *S.aureus* as efficiently as those of control patients. Phagocytosis of pHrodo coated *S.aureus* particles in PMN from JSLE and control patients with JSLE (L) and control (C) serum was measured with flow cytometry (A) and pictures were taken with a confocal microscope (B-F, showing one representative JSLE and one control patient) after a 20 min incubation at 37°C. (A) Fluorescence of PMN was measured using flow cytometry and cells were considered positive if the emitted light was stronger than for cells without particles. N=6 JSLE patients and n=7 control patients were tested. Control PMN without serum or particles are shown in (B). Control PMN with L serum (C) and control PMN with C serum and *S.aureus* (D) showed more cells with particles, than JSLE PMN with L serum (E) or JSLE PMN with C serum and (F) *S.aureus* which instead had more and brighter particles per cell. Blue shows DAPI staining of nuclei, arrows indicate phagocytosed *S.aureus* (red) and conditions were compared with a Kruskal-Wallis-test.

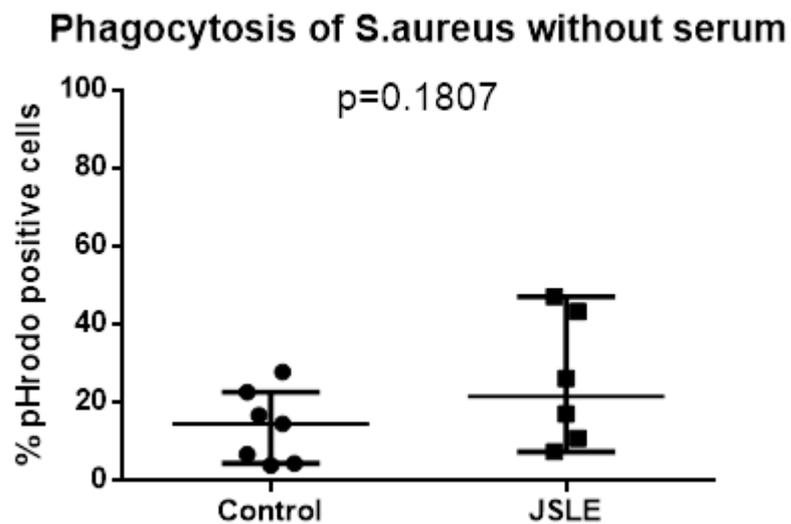


Figure 4.16: Neutrophils of JSLE patients phagocytose more *S.aureus* particles than healthy paediatric control patients when no serum is present. Phagocytosis of pHrodo coated *S.aureus* particles without any serum after 20 min at 37°C was measured with flow cytometry for n=7 healthy paediatric control patients and n=6 JSLE patients. Comparing the two groups with a Mann-Whitney U test there was no significant difference between the two groups. Nevertheless, a trend of p=0.18 for a higher uptake in JSLE patients was observed.

4.5.5.2 Phagocytosis of E.coli by neutrophils

Phagocytosis of E.coli particles by neutrophils of JSLE patients compared with controls was higher but not significantly different ($p=0.08$) (**Figure 4.17**). PMN of JSLE patients showed increased uptake equally with JSLE and control serum, compared to control cells with either JSLE or control serum.

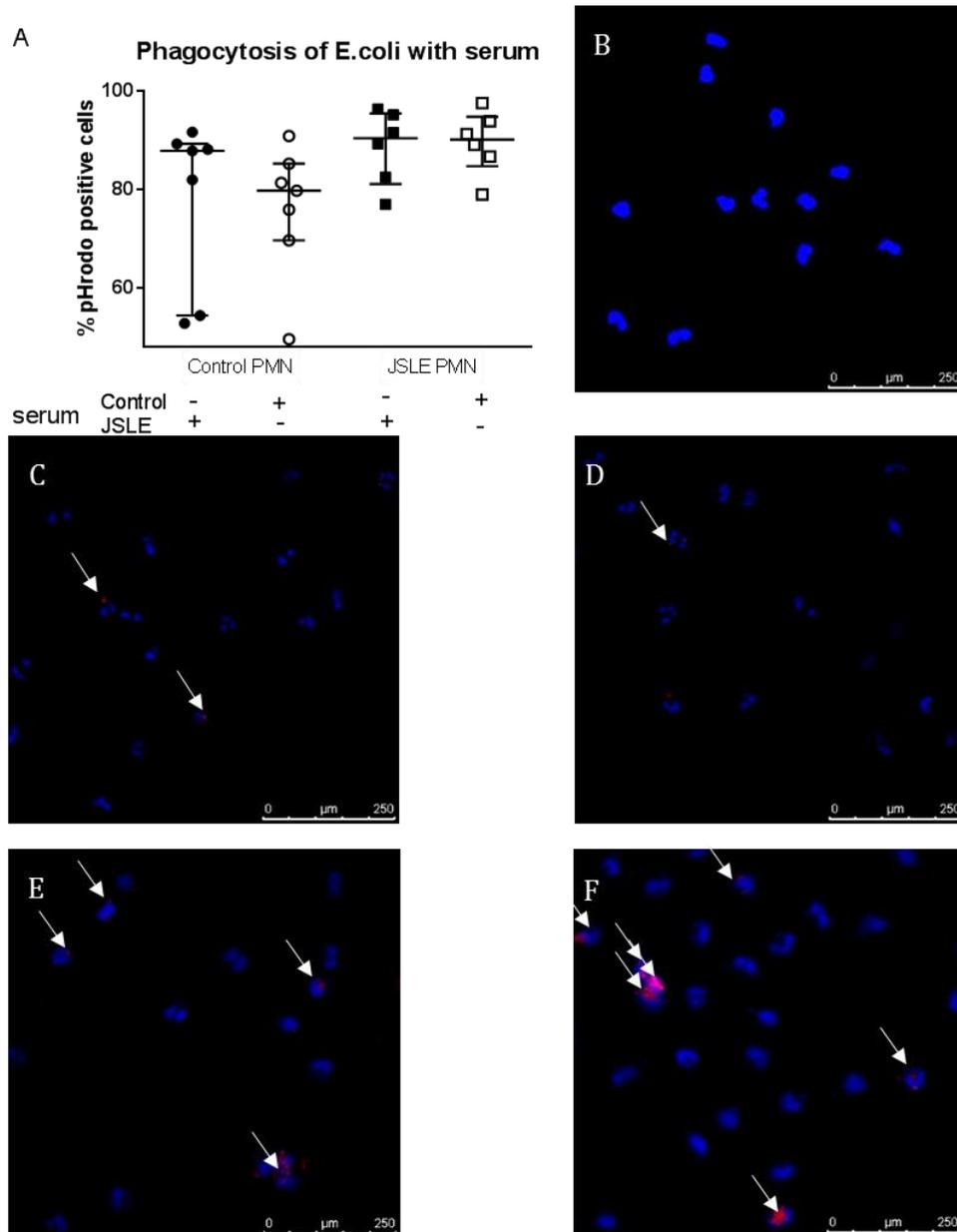


Figure 4.17: Uptake of *E.coli* by neutrophils of JSLE and control patients. Phagocytosis of pHrodo coated *E.coli* particles in PMN from JSLE and control patients with JSLE (L) and control (C) serum was measured with flow cytometry (A) and pictures were taken with a confocal microscope (B-F, showing one representative JSLE and one control patient) after a 20 min incubation at 37°C. (A) Fluorescence of PMN was measured using flow cytometry and cells were considered positive if the emitted light was stronger than for cells without particles. N=6 JSLE patients and n=7 control patients were tested, but only a trend of $p=0.08$ found JSLE neutrophils to be more effective in phagocytosis. JSLE PMN without serum or particles are shown in (B). Control PMN with L serum and *E.coli* (C) or with C serum and *E.coli* (D) showed lower uptake of particles, than JSLE PMN with L serum and *E.coli* (E) and JSLE PMN with C serum and *E.coli* (F) showed more uptake than). Arrows indicate ingested *E.coli* (red), blue shows nuclei with DAPI staining. Results of flow cytometry were compared with a Kruskal-Wallis-test.

4.5.5.3 Phagocytosis of zymosan by neutrophils

Similar to *E.coli*, zymosan particles observed phagocytosis in JSLE PMN was higher at 20 min than in healthy paediatric control patient neutrophils (**Figure 4.18**). Statistical analysis using the Kruskal-Wallis test showed a significant difference between all groups with a p-value of $p=0.04$, but after adjustment for multiple comparison, there is only a trend ($p=0.07$) between control PMN with JSLE serum and JSLE PMN with control serum. Control PMN with control serum and JSLE PMN with control serum have a p-value of 0.28. Results further suggested that for zymosan the control serum had a beneficial effect on uptake even at this short incubation time.

Comparison with lupus serum had been conducted before and it had been demonstrated that control serum can restore phagocytosis in JSLE PMN. The novelty in our data is the investigation of the phagocytic ability of JSLE neutrophils compared to healthy paediatric control patients PMN in a healthy environment. The important comparison is therefore between control and JSLE PMN incubated with control serum. There was significantly higher uptake observed of *E.coli* particles ($p=0.04$) and non-significantly higher uptake of zymosan particles ($p=0.05$) in JSLE PMN compared to healthy paediatric control patients PMN (**Figure 4.19**). *S.aureus* particles did not show a difference in phagocytosis.

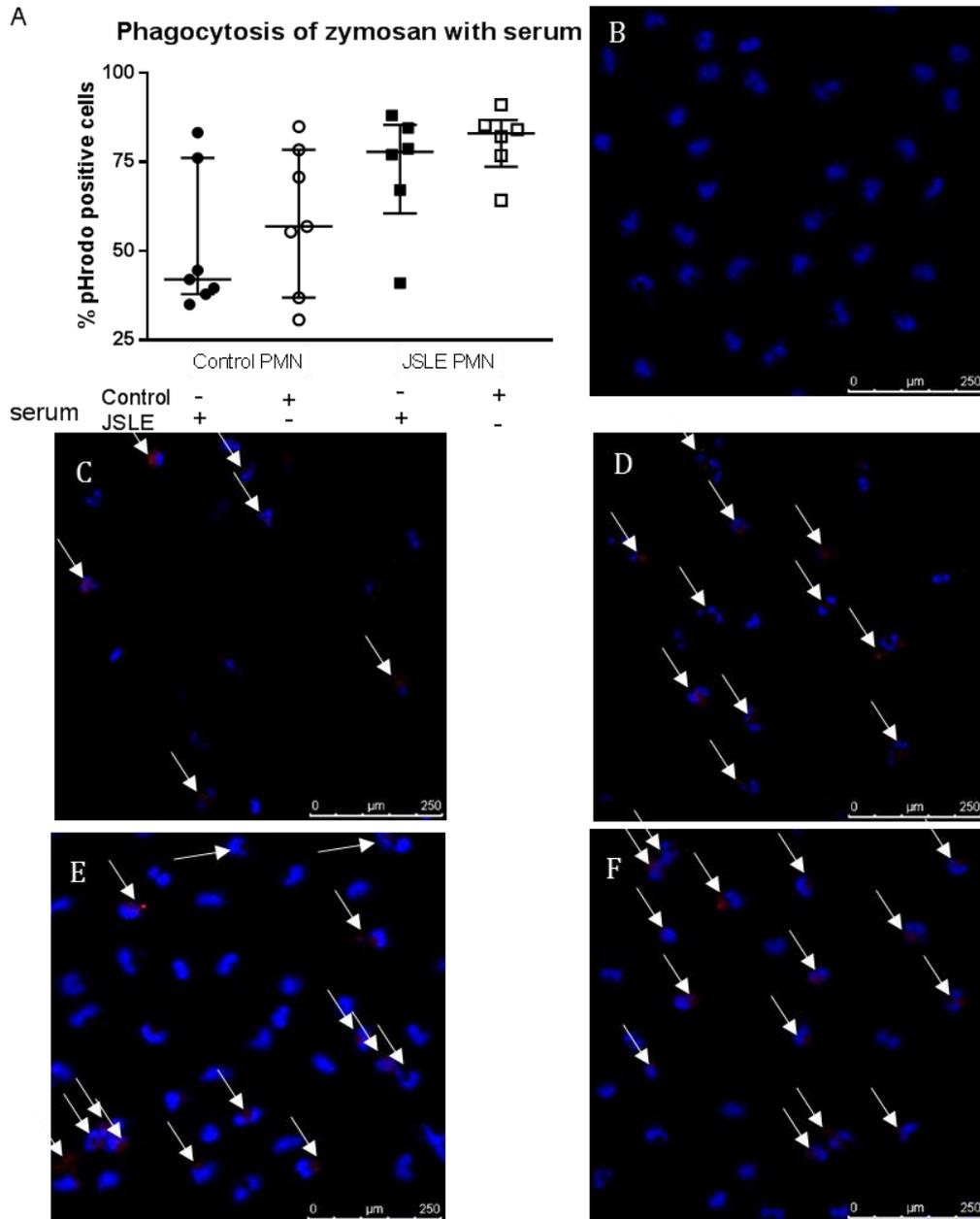


Figure 4.18: Increased uptake of zymosan for JSLE PMN compared to PMN of healthy control patients. Phagocytosis of pHrodo coated zymosan particles in PMN from JSLE (n=6) and control patients (n=7) with JSLE (L) and control (C) serum was measured after a 20 min incubation at 37°C with flow cytometry and found to be significantly different ($p=0.04$) between the groups (A) Fluorescence of PMN was measured using flow cytometry and cells were considered positive if the emitted light was stronger than for cells without particles. Pictures were taken with a confocal microscope (B-F, showing one representative JSLE and one control patient). JSLE PMN without serum or particles were negative for uptake (B). Control PMN with L serum (C) and C serum (D) showed a trend for lower uptake of zymosan than control PMN with L serum (E) or C serum (F). Blue represents DAPI, a DNA staining dye and red indicated ingested zymosan. Arrows indicating zymosan and conditions in flow cytometry were compared with a Kruskal-Wallis-test.

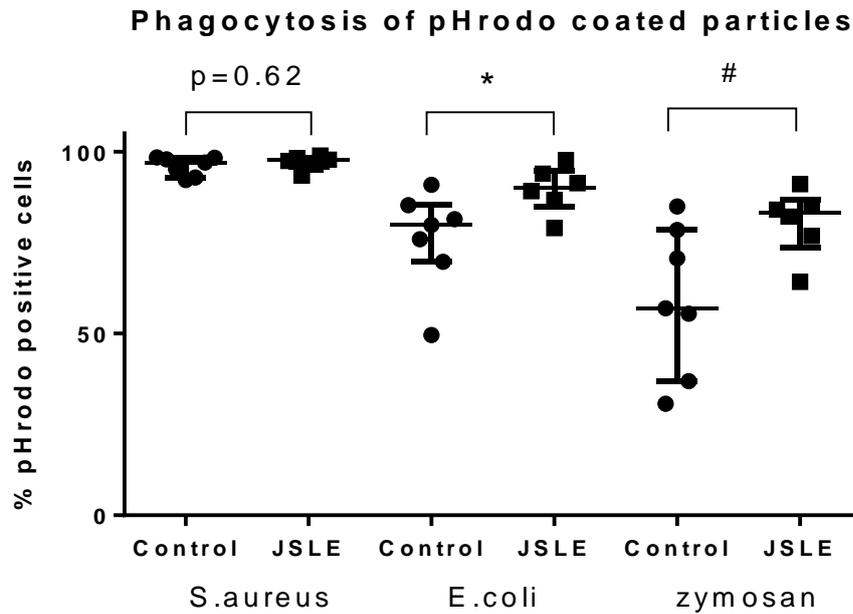


Figure 4.19: Comparison of phagocytic ability of JSLE and healthy paediatric control patients PMN with control serum measured with pHrodo coated bioparticles. PMN of JSLE and healthy paediatric control patients were incubated with control serum and *S.aureus*, *E.coli* and zymosan pHrodo coated bioparticles for 20 min at 37°C. Emitted fluorescence was measured using flow cytometry. # p=0.05, * p<0.05 using Mann Whitney test

4.6 Discussion

In previous studies, an IFN gene signature was described for different inflammatory diseases including JSLE with 82% of these individuals showing a IFN high score [57]. In the JSLE cohort in this study, the IGS was found to be upregulated in the majority of patients. Of the ones tested for their IGS only five out of 19 were IFN low and 74% were classified as IFN high. Rice *et al* (2017) had selected IFI27, IFI44L, IFIT1, ISG15, RSAD2 and SIGLEC1 for their IFN-induced gene signature. From their published panel of genes there was an overlap with this present study, as IFI44L was included in this thesis as well. Initial experiments conducted by our group showed high expression additionally for OAS2, IFI6 and LY6E in JSLE patients, and therefore these genes were included in the IGS panel. LY6E for example has also been used in other studies testing the prevalence of IFN type I in patients with Sjögren's syndrome [247]. In this present study, IFI6 did not correlate with OAS2, IFI44L or LY6E when comparing IFN-induced genes over time. It was therefore excluded from subsequent evaluation as to whether patients remained IFN high or low. IFI6 may be influenced by different or additional factors compared to the other genes. When using the panel of three genes (OAS2, IFI44L and LY6E) no changes in the IFN subtype were observed over time. This is consistent with data from Lindau *et al.* [111] proposing that there are IFN α responders and non-responders who are predefined if they react to stimuli.

Besides the IGS itself, further differences between IFN high and low patients were identified within the transcriptomics data arising from our group. For this reason, investigation of genes coding for proteins related to repair or modification of DNA and phagocytosis related genes was conducted.

BRCA2 and TREX1 were the only genes noted to be different between JSLE and healthy paediatric controls that were due to high expression in IFN high patients. Interestingly, loss of TREX1 was described to trigger systemic autoimmunity with IFN production as cells cannot cope with damaged DNA [248] and mutations in this gene have been described for SLE [42]. Increased expression found in IFN high patients may indicate increased need for nuclease activity processing ssDNA or compensation for a faulty protein.

BRCA2 is not studied as a gene for lupus, but usually considered important in cancer development. Higher expression has been associated rather with breast cancer tissue with 36.6% of breast cancer patients having developed autoantibodies against BRCA2 while only 0.7% of healthy controls had autoantibodies [249].

BRCA2 together with TREX1 may indicate high DNA instability with increased DNA digestion potentially leading to increased apoptosis as it has been observed in JSLE [137].

PRGs were also found to be significantly different between JSLE and healthy paediatric control patients, as well as between the JSLE IFN subgroups themselves.

Phagocytosis has been shown to be dysregulated in both juvenile-onset and adult-onset SLE. Receptors of monocytes are differentially regulated in both groups and result in a decreased phagocytosis of apoptotic neutrophils [4], [169]. Furthermore the uptake of *E.coli* was impaired in SLE, as serum of patients can affect ingestion of *E.coli* particles by neutrophils of SLE [170] and by macrophages of JSLE patients [4]. Since neutrophils are the most abundant phagocyte in blood, it was important to establish if JSLE neutrophils express mRNA and protein of phagocytosis related proteins and if they are intrinsically capable of phagocytosis.

For mRNA expression of AnxA3, CamK1D, CR3 and Dectin-1 there was no significant difference observed between JSLE and healthy paediatric control patients. TLR2 mRNA expression was significantly increased, which has also been shown to occur in PBMCs from SLE patients [250]. Even though they did not stratify patients for IFN high and IFN low, they did look at LY6E and IFN α mRNA expression. Their results indicated no significant ($p=0.089$) positive correlation between IFN α and TLR2. This supports our results with IFN low patients not being significantly lower than IFN highs. Similar to TLR2, in our study S100A9 was significantly increased in JSLE patients and higher with a stronger IFN signature. So far mRNA expression of S100A9 has not been investigated in neutrophils in JSLE, but it has been demonstrated that LDG in

SLE patients highly express S100A9 mRNA. This group also showed that protein expression of S100A9 in the PBMC fraction which contains LDGs correlates with measured plasma levels of S100A8/S100A9 which were significantly higher in SLE than in control patients [251]. As for this thesis, neutrophils were isolated with a density gradient and increased S100A9 expression was measured only in normal density neutrophils. S100A9 is bioactive when it forms a complex with S100A8. This tetrameric form of S100A8/S100A9, also called calprotectin, was shown to be increased in the serum of patients in the cohort studied in this thesis and was also found in serum from SLE patients with cardiovascular disease [252] and in patients with more active disease [253]. No study of the IGS of JSLE patients have investigated the role of S100A9 and TLR2. Interestingly, IFN low patients from our cohort were characterized by significantly higher TLR2 protein expression than the control group whereas only a non-significant increase was observed for the IFN high group. Serum levels for S100A8/S100A9 for IFN low patients were higher than from control and IFN high patients, but did not reach significance.

For both S100A9 and its bioactive form a wide spread has been observed in the IFN low group. Medication seems not to have any contribution to this observation as patients who donated blood for flow cytometry analysis were all on the same drugs. Similarly, serum samples used for ELISA were from patients who all received hydroxychloroquine and mycophenolate. Four of the five patients were additionally on prednisolone, therefore also not explaining the separation of two from the other three patients as seen in **Figure 4.13C**. If looking at S100A9 protein expression IFN low patients with a high disease activity are low and patients with a low disease activity display high protein expression. However, this is not found in JSLE IFN high patients and no correlation is found between disease activity and protein measured with ELISA. It remains therefore questionable if there is a link between disease activity and presence of S100A9 in neutrophils or S100A8/S100A9 in serum. The observed connection may be a coincidence due to the very low number of JSLE IFN low patients.

Upregulation of TLR2 might be due to the apoptotic environment which the neutrophils experience. HMGB-1, also called amphoterin, is for example released from necrotic cells and can trigger TLR2 signalling [254]. S100A9 is generally seen as a protein of inflammation and its expression was shown to be upregulated upon stimulation with LPS representing a bacterial infection [255].

TLR2 and S100A9 mRNA and protein levels were both significantly increased for JSLE compared to control patients. For Fc γ RIIIb on the other hand, only when splitting the JSLE group into IFN high and IFN low a significant increase was observed. High expression was linked to JSLE patients with an elevated IFN signature. Protein expression of Fc γ RIIIb, CD16b, was not significantly increased for IFN high patients. To our knowledge, there has been no paper published with data of Fc γ RIIIb mRNA expression. It has been shown that copy number variations and polymorphisms are related to SLE susceptibility and suggested that an impaired function due to a gene alteration could be the explanation for this relation [172]. Increased mRNA expression could be a result of trying to compensate for a dysfunctional receptor, but also a result of IFN α as it was only seen in the IFN high patients.

The influence of mRNA and protein expression was measured with functional assays comparing neutrophil phagocytosis in JSLE patients and controls. There was no difference in uptake of *S.aureus*, a trend for an increased ingestion of *E.coli* and a significant increase for zymosan uptake, which could be explained by the observed upregulation of TLR2. Phagocytosis of *S.aureus* might have already reached saturation and because of that no difference can be detected. We therefore looked at phagocytosis without serum and found a similar trend to *E.coli*. Denny *et al.* demonstrated in their study in 2010 that LDGs of SLE patients emit significantly less fluorescence when incubated with pHrodo coated *S.aureus* particles. Fluorescence released from normal density granulocytes was also decreased, but non-significantly [145]. The results are not comparable with our study as the incubation with particles and autologous serum was for two hours and effects can be biased by the serum present as JSLE serum has been shown to reduce phagocytosis [4]. Denny *et al.* also looked at the mean fluorescence which for pHrodo results from both the amount of

particles ingested and the acidity in the phagosome. Consequently, a decrease in fluorescence could also indicate a more alkaline pH in the JSLE neutrophils. Another study suggested that there is no difference in the uptake of *E.coli* and *S.aureus*-particles in JSLE patients, but did not indicate if, serum (and if so, what type) was used and they also did not state how long cells were incubated with particles [238]. Saturation might have occurred in the latter study, as observed with *S.aureus* in the data reported in this chapter.

Functional phagocytosis assays reported in the literature are difficult to compare and it is crucial to know all exact timings and parameters. In our case, functional assays show JSLE neutrophils to be at least as efficient as control neutrophils, when the effect of serum is diminished with a short incubation without preopsonisation. This is also supported, as gene and protein expression in JSLE patients show a more activated profile for phagocytosis compared to control patients. Important receptors, like TLR2 and FcγRIIIb, and stimulatory molecules, like S100A9, are upregulated and can explain the observed trend for even more efficient uptake of *E.coli* and zymosan in JSLE.

4.7 Conclusion

JSLE IFN high and IFN low patients differ as hypothesized not just in their IFN signature in neutrophils, but also regarding expression of phagocytosis-related genes and to a lesser extent expression of genes involved in DNA modification. Furthermore, the increased expression of PRG in JSLE patients compared to healthy paediatric controls was observed to be translated into protein. The difference between JSLE IFN high and JSLE IFN low patients was less clear than observed for mRNA expression. Upregulated expression of PRG was visible in phagocytosis assays as neutrophils of JSLE patients showed enhanced uptake of bioparticles compared to neutrophils of healthy paediatric controls. A difference between phagocytic ability between JSLE patients' IFN subtypes has not been observed.

5 The influence of the cell environment on IGS and PRGs in JSLE patients

5.1 Introduction

In Chapter 4, the IGS and PRGs were assessed in neutrophils of JSLE patients and their IFN subtypes compared to those of healthy paediatric control patient PMNs. Studies have been published about the presence of the IGS [56], [57]. However, the causes and influences of this signature are still unknown. Furthermore, the triggers for the PRGs in JSLE patients described in Chapter 4, require further investigation.

Neutrophils in JSLE patients are influenced by many different stimuli. It may therefore be valuable to start the assessment of potential factors influencing these signatures with processes known to be dysregulated in JSLE.

One of these processes is cell death and in particular apoptosis, which should be a non-inflammatory process leading to clearance of dead or dying cells – see Section 1.6.4. This however has been noted to occur at a higher rate and dysregulated manner in JSLE patients, as well as adult-onset SLE patients [137]. Apoptotic cell removal has additionally been found to be deficient in JSLE patients [168]. Uncleared apoptotic cells could release cytokines and increase the amount of cell debris that may stimulate both the IGS and PRGs.

Another process identified as being dysregulated in JSLE is NETosis (also referred to as ‘netting’) and it is considered a cause of disease onset. This type of neutrophil-specific cell death is linked to LDGs in lupus, releases chromatin containing nucleosomes together with antimicrobial proteins and is causing damage to tissue in SLE [237].

Both increased apoptosis and NETosis may lead to the presence of nucleosomes which was suggested to result in increased IFN α production [111]. IFN α itself should induce genes of the IGS and may also affect some PRGs.

Netting LDGs from SLE patients have also been shown to produce higher levels of TNF α compared to healthy control PMN or even compared to SLE PMN [145]. Increased numbers of LDGs in JSLE and increased NETosis suggest TNF α to play a role in diseases and may affect the IGS and PRGs [159], [237].

5.2 Chapter hypothesis

The hypothesis to be investigated is that: Differences between JSLE patients and paediatric control patients in expressing an IGS and a PGS, may be explained by differences in components of the cellular environment of neutrophils, which causes changes in mRNA and protein expression.

5.3 Objectives

The objectives for this chapter are:

Objective 1: To investigate the impact of the apoptotic environment, nucleosomes, IFN α and TNF α on gene and protein expression of PRGs in neutrophils or whole blood.

Objective 2: To measure the influence of the apoptotic environment, nucleosomes, IFN α and TNF α on gene expression of IGS in neutrophils or whole blood.

5.4 Chapter specific methods

5.4.1 Selection of housekeeping genes

As detailed in Section 4.4.2, the GeNorm™ reference gene selection kit from Primerdesign was used when possible with the following primers for testing appropriate housekeeping genes:

18S, ACTB, ATP5B, B2M, CYC1, EIF4A2, GAPDH, RPL13A, SDHA, TOP1, UBC, YWHAZ.

For a comparison of two conditions for example cDNA of neutrophils stimulated with or without apoptotic supernatant, qPCRs with six cDNA samples were performed with three samples per condition.

Results were then analysed with qbasePLUS (Biogazelle), which gives the M- and V-value as results. The M-value expresses variability of each gene between the conditions with a cut-off of 0.5 considered as stable. Genes are presented from the weakest (high M-value) to the strongest gene (lowest M-value) from left to right. The V-value determines how many housekeeping genes are required for optimal normalization. Once the V-value reaches below 0.15 the number of housekeeping genes is considered sufficient. Results are shown in step-wise inclusion of the next most stable gene.

For incubation with apoptotic supernatants healthy adult bead-purified neutrophils were stimulated with OVN medium (plus 10% FCS) or apoptotic supernatant (plus 10% FCS). To take into account the effect of other immune cells a further condition was included which involved 95% neutrophils together with 5% PBMCs stimulated in apoptotic supernatant (plus 10% FCS). All three sample types were tested with GeNorm™ reference gene selection kit (**Figure 5.1**).

Analysis showed that except for RPL13A, GAPDH and EIF4A2, all housekeeping genes had an M-value less than 0.5 which is considered the cut-off for stable gene expression (**A**). The three ideal genes for normalization with apoptotic supernatant were ACTB, UBC and B2M (**B**).

For all other assays, either mRNA from 1 ml whole blood or paediatric control patient neutrophils were used.

Due to the availability of the analysis software and chicken nucleosomes not coinciding, whole blood stimulations were tested with native HEK293-derived nucleosomes. UBC, TOP1 and EIF4A2 were the three most stable target genes, but all genes except for GAPDH were under the M-value of 0.5 and are therefore considered stable (**Figure 5.2 A**). With a V-value of 0.055 three housekeeping genes were suggested as appropriate: UBC, TOP1 and EIF4A2 (**B**). Due to low mRNA values only the two most stable housekeeping genes were used. To

confirm that UBC and TOP1 alone were valid housekeeping genes also with chicken nucleosomes, anti-IFNAR-antibody and isotype control the C_T -values of the different stimulations were compared (**C**). Statistical analysis showed that there was no difference (UBC $p=0.64$; TOP1 $p=0.24$) between the treatments for either of the housekeeping genes and UBC and TOP1 were therefore used for normalization.

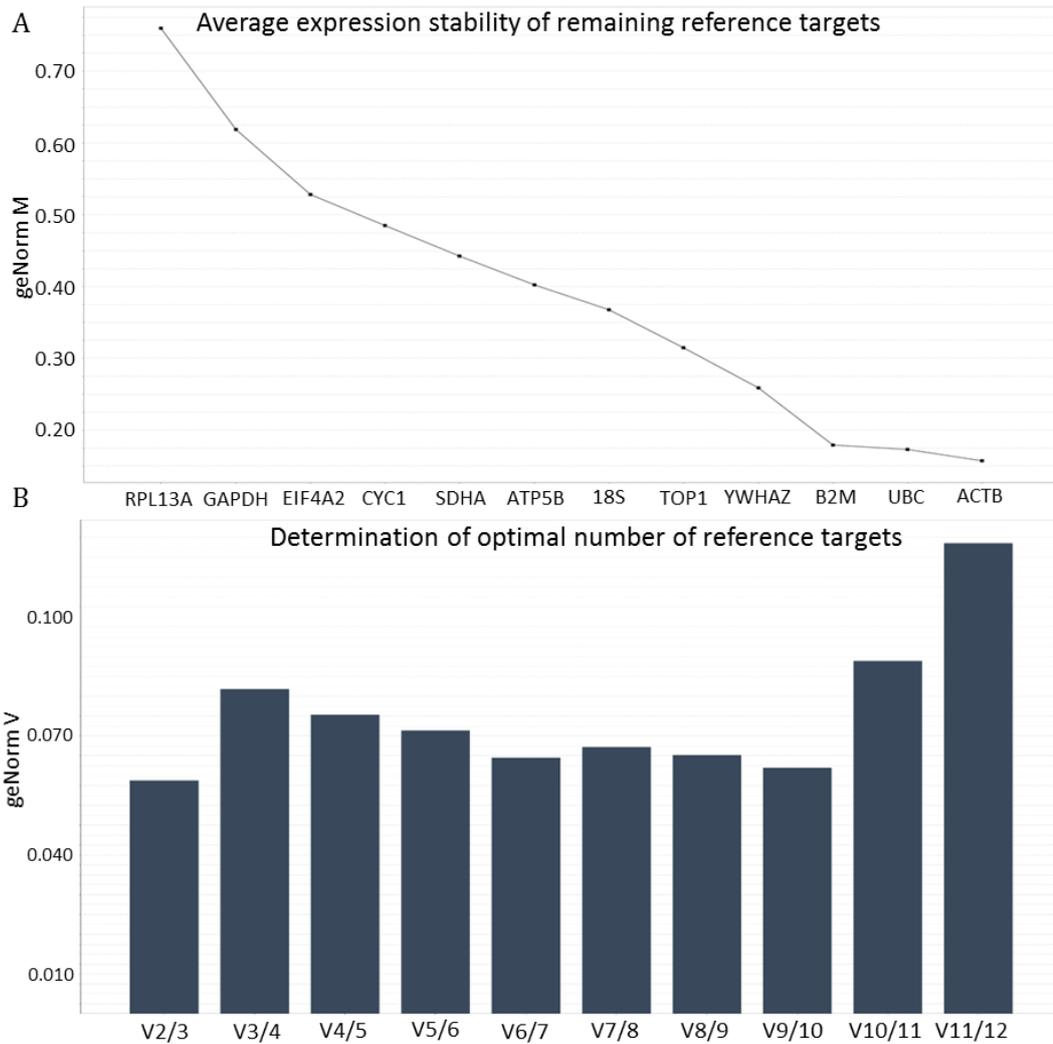


Figure 5.1: Reference target stability and optimal number of housekeeping genes for neutrophil stimulation with apoptotic supernatants. In total nine cDNA samples of bead-purified neutrophils, with or without apoptotic supernatant or neutrophils in the presence of apoptotic supernatant and 5% PBMCs, were tested for their expression of 12 housekeeping genes. Stability of each gene is shown in (A) with the least stable gene on the left and the most stable gene on the right. GeNorm V in (B) shows how many genes are required to reach $V < 0.150$ by performing pairwise variation. A maximum of three genes (ACTB, UBC and B2M) is necessary for normalization.

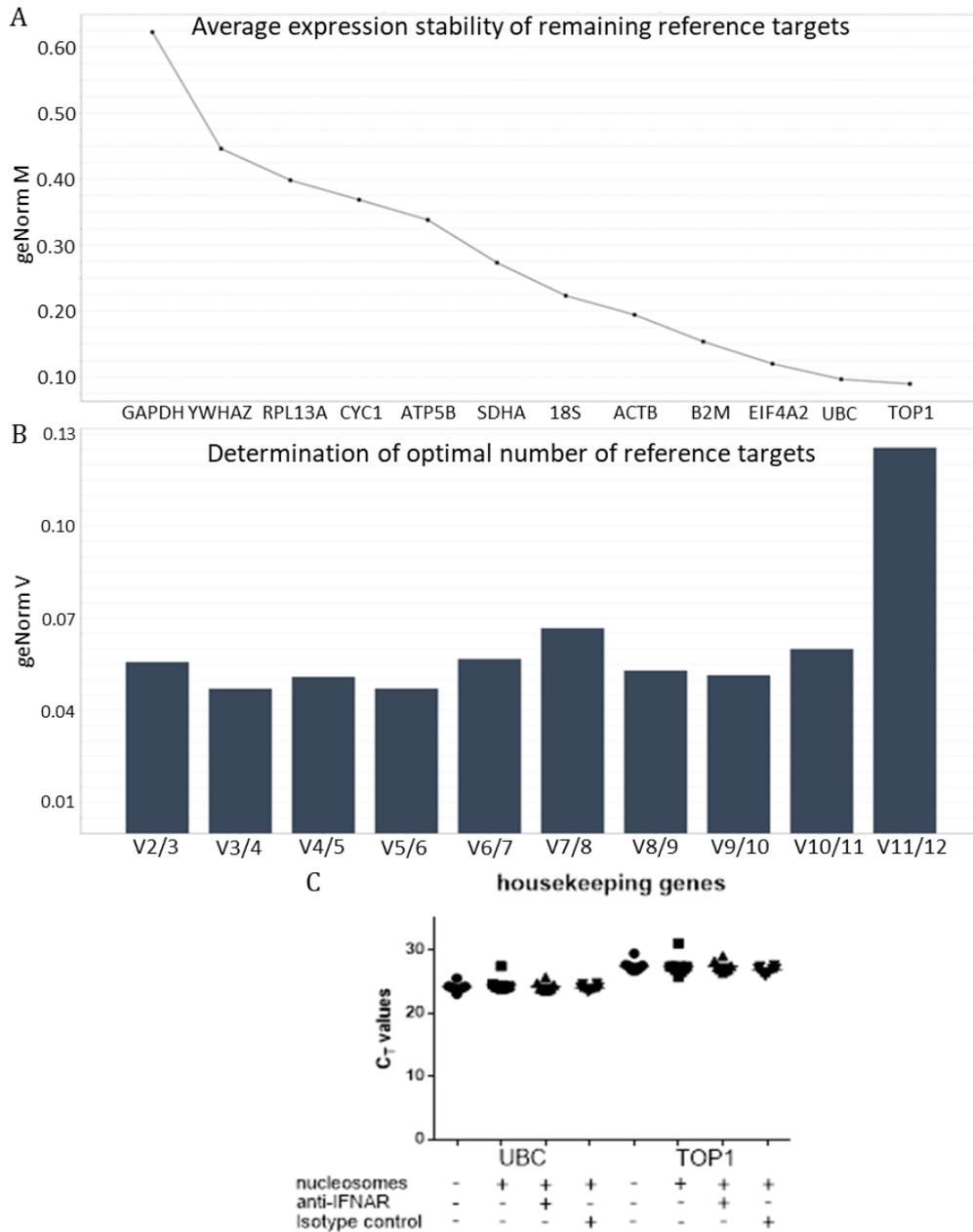


Figure 5.2: Reference target stability and optimal number of housekeeping genes for whole blood stimulations. Three cDNA samples of unstimulated and four cDNA samples of native nucleosome (HEK293-derived; AMS Biotechnology, UK) stimulated healthy adult control whole blood were tested for their expression of 12 housekeeping genes. Stability of each gene is shown in (A) with the least stable gene on the left and the most stable gene on the right. GeNorm V in (B) shows how many genes are required to reach $V < 0.150$ by performing pairwise variation. Three genes (TOP1, UBC and EIF4A2) are sufficient for optimal normalization. UBC and TOP1 were chosen to be used as housekeeping genes and comparison of C_T -values for their expression ($n=8$ for each condition; C) there is also no change of mRNA after stimulation with chicken nucleosomes with or without anti-IFNAR-antibody and with or without Isotype control. Friedman test with Dunn's multiple comparison.

IFN α stimulations of paediatric control patient neutrophils were conducted before the GeNorm™ kit and Biogazelle software were available and ACTB had been chosen as a housekeeping gene. Six cDNAs were run retrospectively comparing all 12 housekeeping genes to evaluate the strength of ACTB as a housekeeping gene.

ACTB was the fourth most stable housekeeping gene with an M-value of 0.03 showing very little variation between the conditions (**Figure 5.3 A**). Additionally, statistical analysis to identify differences between six unstimulated and stimulated samples showed that C_T-values of ACTB does not vary between treated and untreated neutrophils (p=0.16; **B**).

Paediatric control patient neutrophils were stimulated with TNF α , but this was conducted after the Biogazelle software was available. C_T-values of ACTB and TOP1 were therefore compared to establish that there is no variation between the treatments (**Figure 5.4**). Indeed, statistical analysis confirmed that both ACTB (p=0.31) and TOP1 (p=0.19) are not different for TNF α treated or untreated neutrophils.

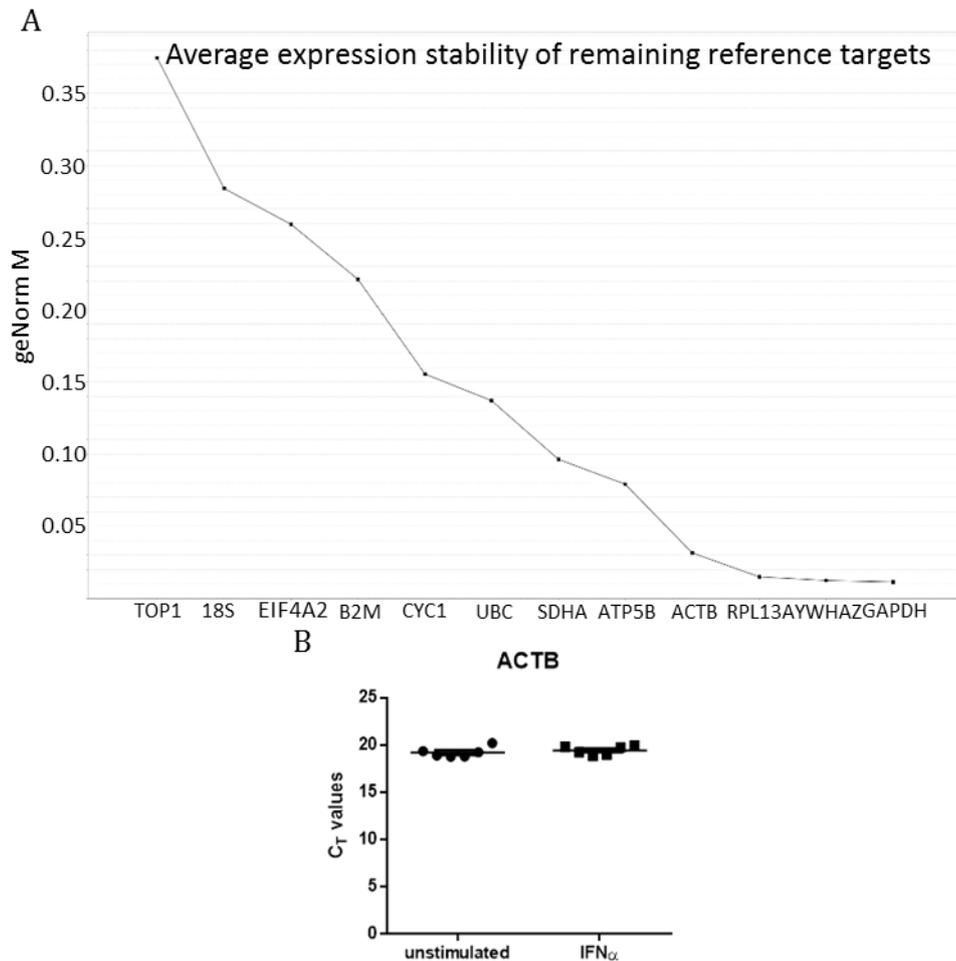


Figure 5.3: Reference target stability and optimal number of housekeeping genes for stimulation of paediatric control patient neutrophils with IFN α . Three cDNA samples each of unstimulated and IFN α stimulated paediatric control patient neutrophils were tested for their expression of 12 housekeeping genes. Stability of each gene is shown in (A) with the least stable gene on the left and the most stable gene on the right. ACTB has an M-value of 0.03 showing strong stability. Comparison of C_T-values for ACTB expression (n=6; B) showed that there is also no change of mRNA after stimulation with IFN α and confirm it to be a very good housekeeping gene. Wilcoxon matched-pairs signed rank test

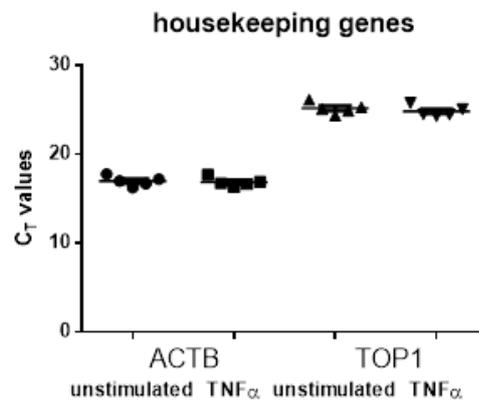


Figure 5.4: mRNA expression of ACTB and TOP1 as housekeeping genes for TNF α stimulation. Paediatric control patient neutrophils were stimulated with 1 ng/ml TNF α and mRNA expression of ACTB and TOP1 were tested with real-time PCR for their expression. No difference was observed either for the C_T-values for ACTB or for TOP1 between the stimulations. Wilcoxon matched-pairs signed rank test

5.4.2 Collecting supernatant from whole blood stimulations for ELISA

For each stimulation, 1 ml of whole blood was used and incubated for 5 h at 37°C as described in 2.2.10.2. Afterwards the blood was mixed with 200 µl HetaSep and left to separate for 30-45 min. 550-600 µl of plasma layer was removed and centrifuged with an additional 2.4 ml PBS. Supernatants were aliquoted and frozen at -80°C until used for ELISA and the pellet was used for further processing for RNA extraction as described in 2.10.3.

5.4.3 Time course of paediatric control patient neutrophils stimulated with IFN α

A time course assay was performed over 8 h (n=3) to assess which time point would be best to investigate changes in protein levels reflecting the increase seen in the mRNA expression. While GM-CSF was already increased after 6 h, the highest difference with least variation between untreated and IFN α stimulated cells was observed at 7 h (**Figure 5.5**). For all subsequent measurements of both intra- and extracellular protein, the 7-hour time point was chosen.

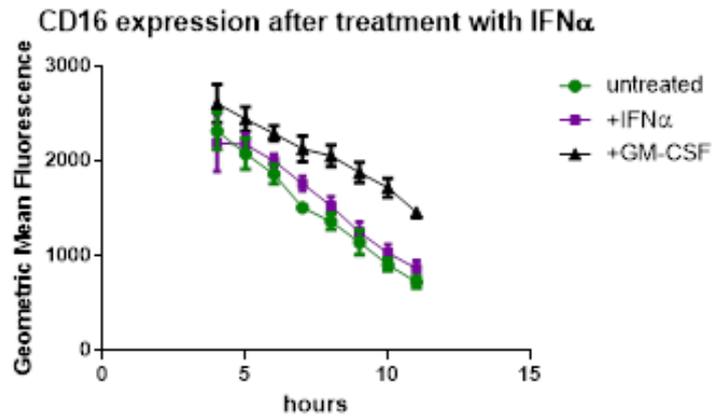


Figure 5.5: CD16b expression after incubation with IFN α and GM-CSF over an 8 h time-course. Neutrophils from three paediatric control patients were stimulated with or without 10 ng/ml IFN α or 5 ng/ml GM-CSF and 10% FCS. After 4 h, CD16b surface expression was measured every hour with flow cytometry for a further 11 h.

5.5 Results

5.5.1 Apoptotic environment and its influence on neutrophils

Increased apoptosis occurs in JSLE patients and may therefore influence the PRG and IGS which were investigated in Chapter 4. To investigate the influence of cytokines released upon apoptosis on PRGs and IGS in neutrophils, we stimulated ultra-pure neutrophils or neutrophils with 5% PBMC in apoptotic supernatant (AS) (plus 10% FCS) or 10% FCS with media as described in 2.2.4.2. Ultra-pure neutrophils were used in this analysis to exclude the presence of IFN α producing cells to allow analysis if apoptotic supernatant alone can change the IGS or PGS. For non ultra-pure cells a cut-off of 5% for PBMCs was chosen to allow production of IFN α while permitting measurement of mostly neutrophil mRNA with downstream qPCR analysis. The two hour time span was primarily to investigate mRNA changes, while at six hours protein expression was expected to change. In order to observe changes in cell death both time points were evaluated. These stimulations were conducted with blood from healthy adult controls due to the high number of PMNs needed. Paediatric control blood would not have been sufficient to compare three conditions with one donor, especially because during the ultra-purification step neutrophils were lost.

5.5.1.1 Influence of apoptotic environment on PRGs of neutrophils

AS did not alter gene expression significantly for TLR2 (**Figure 5.6 A**) or S100A9 (**B**) at 2 or 6 h. TLR2 showed a trend to be increased at 6 h with an overall p-value of 0.08 and a Dunn's multiple comparison p-value of 0.05 in the presence of supernatant and PBMCs compared to medium stimulated ultrapure neutrophils. Expression of Fc γ RIIIb decreased significantly with the stimulation of AS when PBMCs were present after 6 h (**C**). This decrease may result partly from the sole presence of PBMCs as they have no Fc γ RIIIb mRNA, but this result is supported by supernatant alone causing a non-significant decrease in Fc γ RIIIb expression.

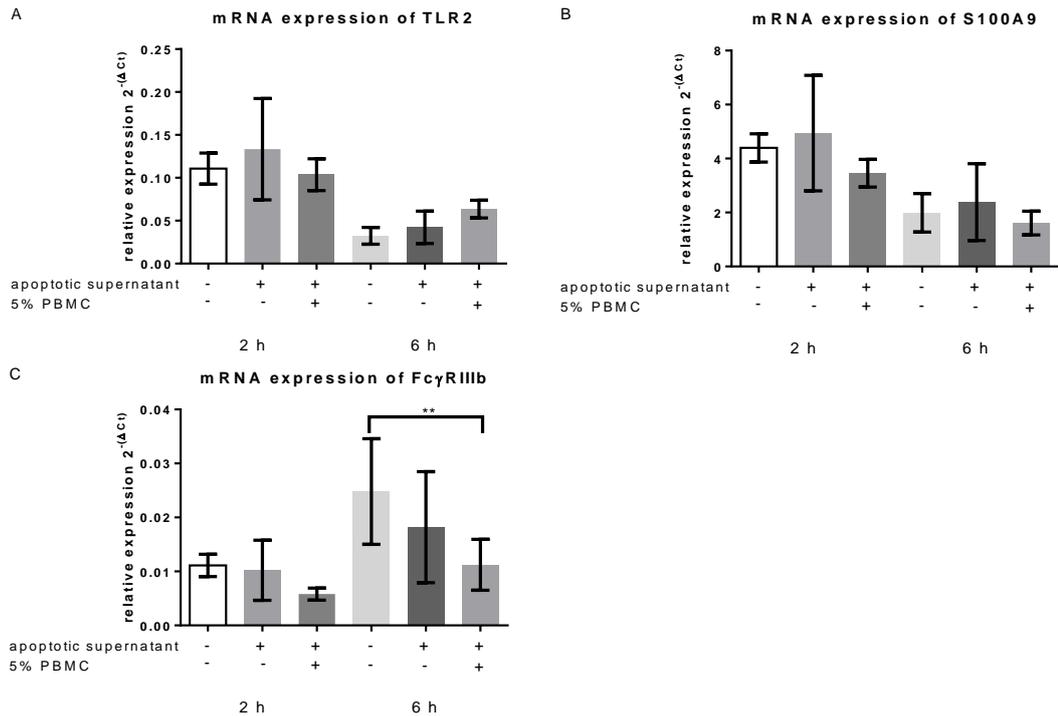


Figure 5.6: mRNA expression of PRGs at 2 h and 6 h for neutrophils in the presence or absence of PBMCs incubated with AS of 1.5×10^6 dying neutrophils. Ultrapure neutrophils (purified with magnetic beads) ($n=5$) were used or as a mixture of 95% neutrophils and 5% PBMCs. They were stimulated with apoptotic supernatant (AS) for 2 h and 6 h. mRNA expression of TLR2 (A), S100A9 (B) and FcγRIIIb (C) was measured with real-time PCR using SYBR green and results were normalized to the geometric mean of UBC, ACTB and B2M. TLR2 only increased non-significantly ($p=0.08$) at 6 h for ANOVA and $p=0.05$ for Dunn's multiple comparison when medium treated PMN were compared with PMN with 5% PBMC and AS. There was no change in the expression of S100A9, but a significant difference for FcγRIIIb comparing medium treated PMN with PMN with 5% PBMC and AS. ** $p<0.01$, Friedman test with Dunn's multiple comparison

At the 6 h time point, protein expression was also measured, but as observed for the mRNA expression there were no changes in TLR2 or S100A9 (**Figure 5.7 A+B**). For both intra- and extracellular CD16b protein there was no difference observed with incubation of AS in the absence or presence of PBMCs (**C+D**). This was in contrast to the findings on mRNA levels which had suggested a decrease of FcγRIIIb with AS in the presence of PBMC. While there was also no difference in released S100A9, CD16b was significantly shed more ($p=0.02$) into the supernatant in the presence of apoptotic supernatant and PBMCs. Ultrapure neutrophils did not show a significant release.

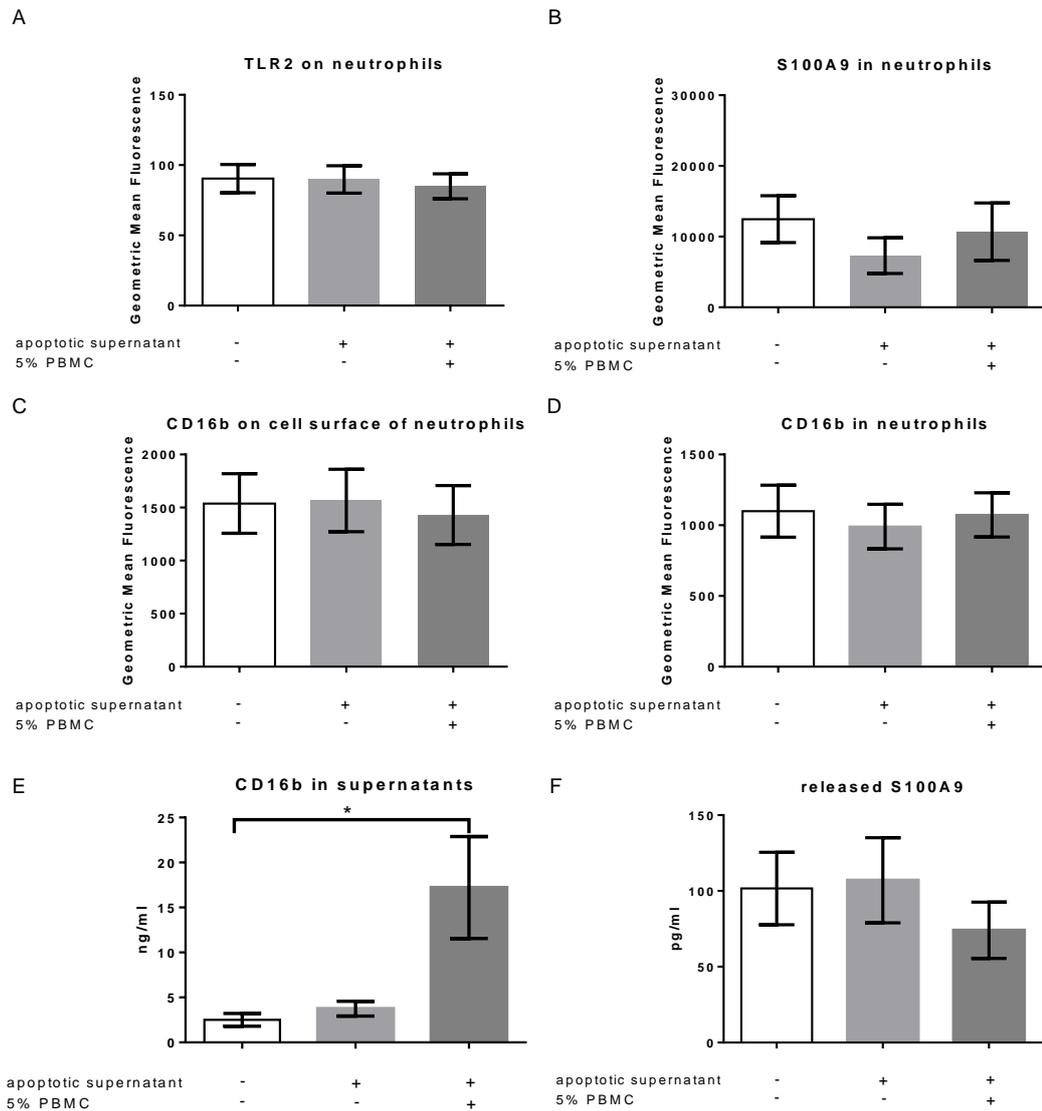
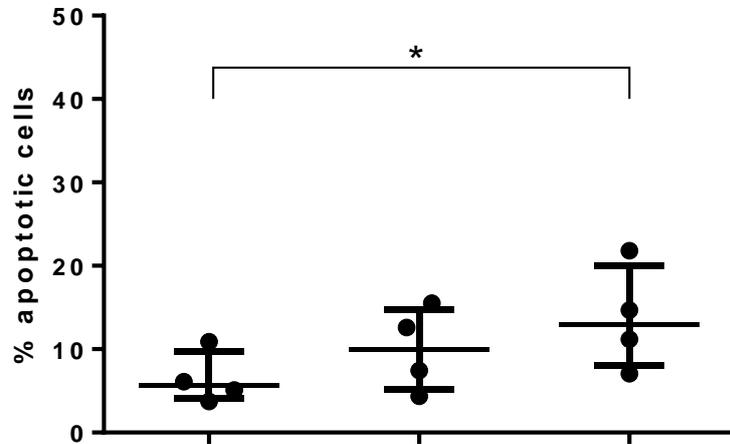


Figure 5.7: Protein expression of PRGs after stimulation with apoptotic supernatant (AS). Neutrophils (n=5) were stimulated either as ultrapure (purified with magnetic beads) or as a mixture of 95% neutrophils and 5% PBMCs with AS for 6 h. Protein of TLR2 (A), S100A9 (B) and CD16b (C+D n=3) was measured with flow cytometry. Release of S100A9 (F) and CD16b (E) was additionally measured in the supernatants. TLR2, S100A9, CD16b and released S100A9 did not show changes for their expression. Only shed CD16b increased significantly in the presence of PBMCs and AS (p=0.02). * p<0.05, Friedman test with Dunn's multiple comparison

Neutrophils shed CD16b when they undergo apoptosis (Dransfield et al., 1994): an increase in CD16b may suggest apoptotic cells. Indeed, at 2 h there is a non-significant increase of apoptotic cells for incubation with supernatant and a significantly higher percentage of apoptotic cells observed for incubation with apoptotic supernatant in the presence of PBMCs (**Figure 5.8 A**). There were no differences in apoptosis observed at 6 h (**B**). Cell death at 2 h may influence the presence of shed CD16b. PMN incubated with PBMC were not purified with magnetic beads before incubation, this process might have removed cells that were apoptotic in the other two conditions. Nevertheless, ultrapure PMNs incubated with supernatant indicate increased apoptosis at 2 h.

A

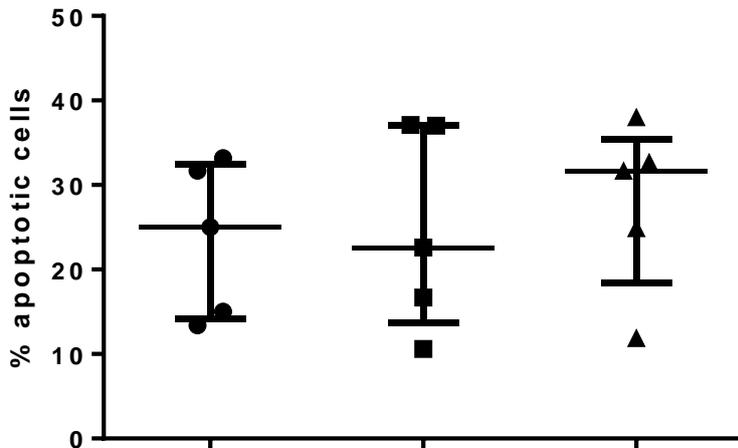
apoptotic PMN 2 h



apoptotic supernatant - + +
5% PBMC - - +

B

apoptotic PMN 6 h



apoptotic supernatant - + +
5% PBMC - - +

Figure 5.8: Apoptosis of PMN in the presence or absence of apoptotic supernatant and PBMCs at 2 h and 6 h. Cell death was measured with flow cytometry using Annexin V and PI for ultrapure neutrophils (purified with magnetic beads) or as a mixture of 95% neutrophils and 5% PBMCs with AS at 2 h (n=4) and 6 h (n = 5). A significant difference was observed at 2 h comparing medium treated-PMN with neutrophils treated with AS in the presence of 5% PBMCs. There was no difference at 6 h. * p<0.05, Friedman test with Dunn's multiple comparison

5.5.1.2 Influence of apoptotic environment on IGS in neutrophils

With regards to the PRGs, only CD16b was changed after exposure to apoptotic supernatant and only in the presence of PBMC (mRNA in **Figure 5.6** and protein in **Figure 5.7**). For the IGS, OAS2 was significantly increased for incubation of neutrophils with apoptotic supernatant in the presence of PBMCs compared to the untreated PMNs ($p=0.02$, **Figure 5.9 A**). IFI44L expression was not significantly different between the groups ($p=0.09$, **B**), whereas LY6E showed a significant overall difference with Friedman's test ($p=0.02$, **C**), but only showed a non-significant adjusted $p=0.05$ with Dunn's multiple comparison for PMN with apoptotic supernatant in the presence of PBMCs compared to the unstimulated PMNs.

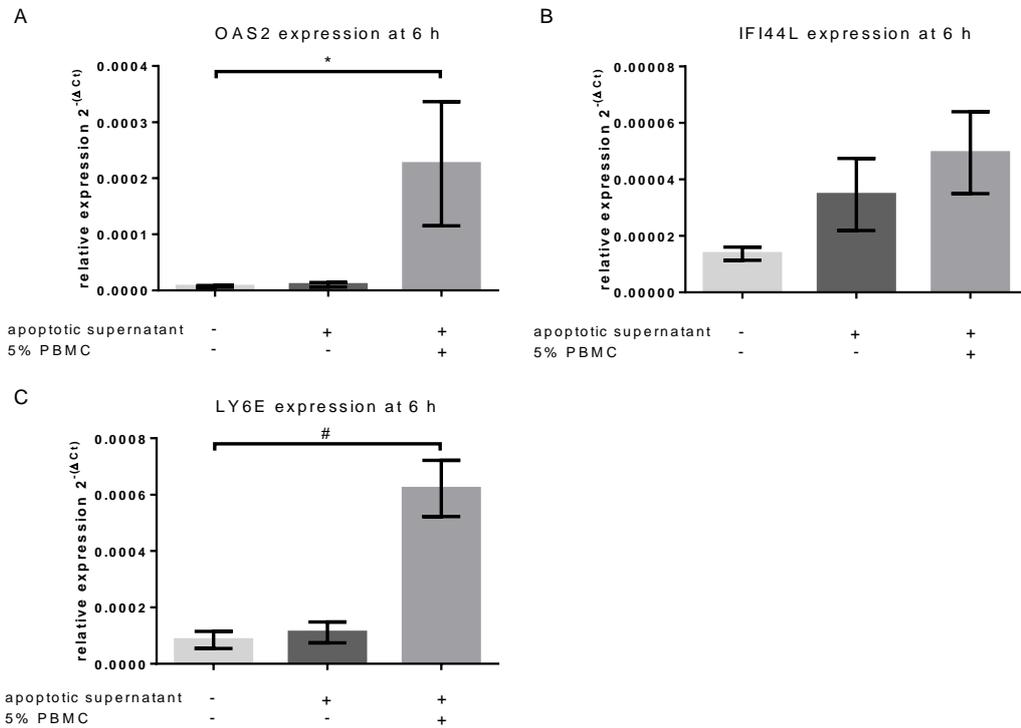


Figure 5.9: IGS induction of healthy adult control PMN with apoptotic supernatant in the presence of PBMC. mRNA expression of IGs was measured in ultrapure PMN with or without AS or PMN with 5% PBMCs and AS (n=5 each) after 6 h using real-time PCR with SYBR green. Results are shown as relative expression normalized to the geometric mean of three housekeeping genes (UBC, ACTB and B2M). A significant increase of OAS2 expression was observed for PMN which were incubated together with PBMCs and AS compared to untreated ultrapure PMN. PMNs with PBMCs stimulated with AS increased the expression for IFI44L only non-significantly (p=0.09). For LY6E a significant difference between the groups was observed (p=0.02) with a non-significant difference for AS stimulated PMN with PBMCs compared to unstimulated PMN. #=0.05; * p<0.05 Friedman test, Dunn's multiple comparison

5.5.2 The impact of nucleosomes

Nucleosomes, present in the JSLE environment in cell debris and NETs, contain both protein and DNA and are strong stimuli for antibody development. Whole blood was therefore stimulated with nucleosomes isolated from chicken blood as described in 2.2.10. After 5 h of incubation, mRNA and protein was measured to investigate the influence on PRGs and IGS.

5.5.2.1 PRGs in the presence or absence of nucleosomes in whole blood

Both TLR2 and S100A9 mRNA levels were not influenced significantly by the presence of nucleosomes, but a 31.3% and 15.8% decrease ($p=0.07$, $p=0.19$ respectively, **Figure 5.10 A+B**) was observed. Nucleosomes significantly reduced Fc γ RIIb expression in whole blood after 5 h (**C**).

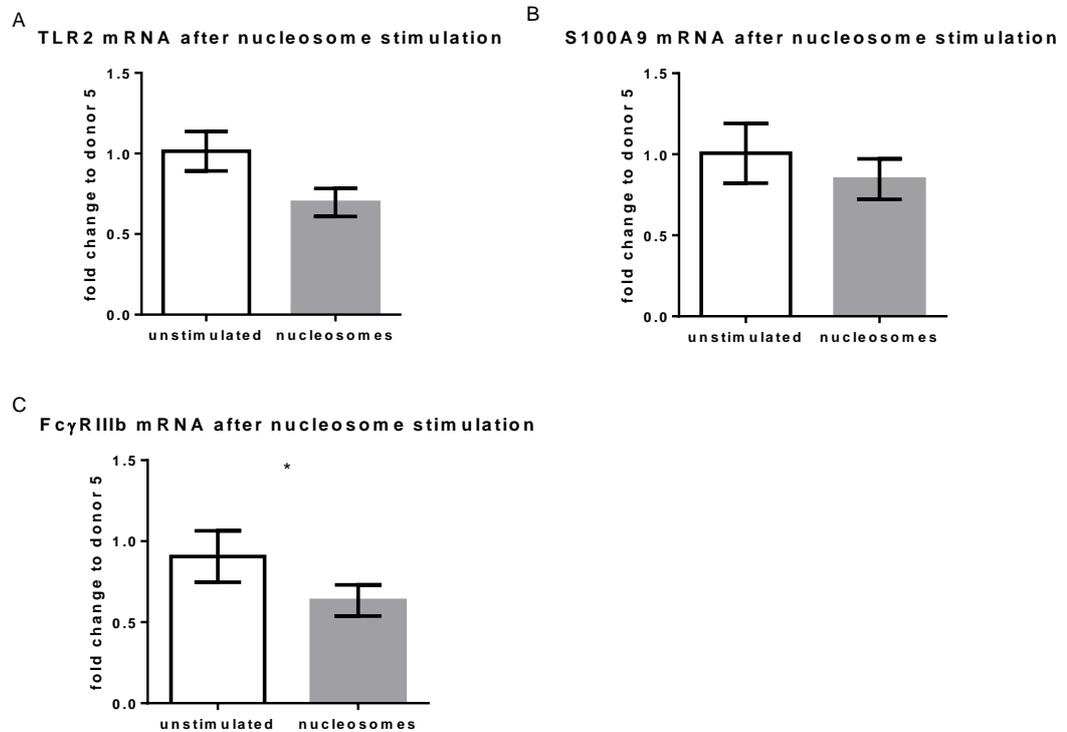


Figure 5.10: mRNA expression of TLR2, S100A9 and FcγRIIIb in whole blood after 5 h stimulation with nucleosomes. Whole blood (n=8) was stimulated with nucleosomes for 5 h or was left untreated and mRNA expression was measured with real-time PCR using SYBR Green. Target genes were normalized to the geometric mean of UBC and TOP1. Results are shown as fold change to donor 5. TLR2 (A) and S100A9 (B) did not show a significant difference although a decrease was observed in TLR2 (p=0.07). FcγRIIIb (C) was significantly decreased in whole blood stimulated with nucleosomes. *p<0.05 paired t-test after normality confirmation with D'Agostino & Pearson omnibus normality test

Protein expression was measured after whole blood stimulation of neutrophils from healthy adult controls with or without nucleosomes in the presence of Brefeldin A using flow cytometry (intracellular and cell surface staining). Supernatants for the S100A8/S100A9 ELISA were obtained from stimulations with or without nucleosomes without Brefeldin A as described in Section 5.4.3. Protein expression (**Figure 5.11**) of phagocytosis-related genes TLR2 and S100A9 in neutrophils showed no changes after nucleosome stimulation for 5 h (**A+C**). While TLR2 protein levels in neutrophils (**B**) also remained unaltered, PBMCs had significantly higher S100A9 protein expression ($p=0.048$; **D**). The bioactive form of the S100A8/S100A9 protein was significantly increased after nucleosome stimulation ($p=0.039$; **E**). Nevertheless, S100A9 within this complex may result from both neutrophils as well as PBMCs, because Brefeldin A prevented the release of S100A9 in **C** and **D**. CD16b protein expression followed the observed changes in mRNA expression with a significant decreased level of extracellular protein expression ($p=0.006$), but unchanged intracellular CD16b. This would suggest shedding of protein into the supernatant. CD16b expression in the supernatant was measured using ELISA (data not shown), but assay technical issues stopped further exploration. There was a strong variation apparent between the replicates. Supernatants were frozen with HetaSep and PBS and some precipitate had built up when they were defrosted. Within the kit description it is mentioned, that precipitates may cause interference.

These results suggest only PMN involvement for CD16b release, but comparison of granularity of neutrophils reveals a significant decrease with nucleosome stimulation ($p=0.03$; **Figure 5.12 A**). This was assessed by gating the neutrophil population with forward scatter (FSC) and sideward scatter (SSC). Compared to unstimulated PMN (**B**), nucleosome stimulation (**C**) causes the neutrophil population to drop for the SSC which represents a decrease in granularity. This may suggest that granules are released upon stimulation with nucleosomes. This process, called exocytosis, can release proteins such as antimicrobials, hydrolases and proteinases.

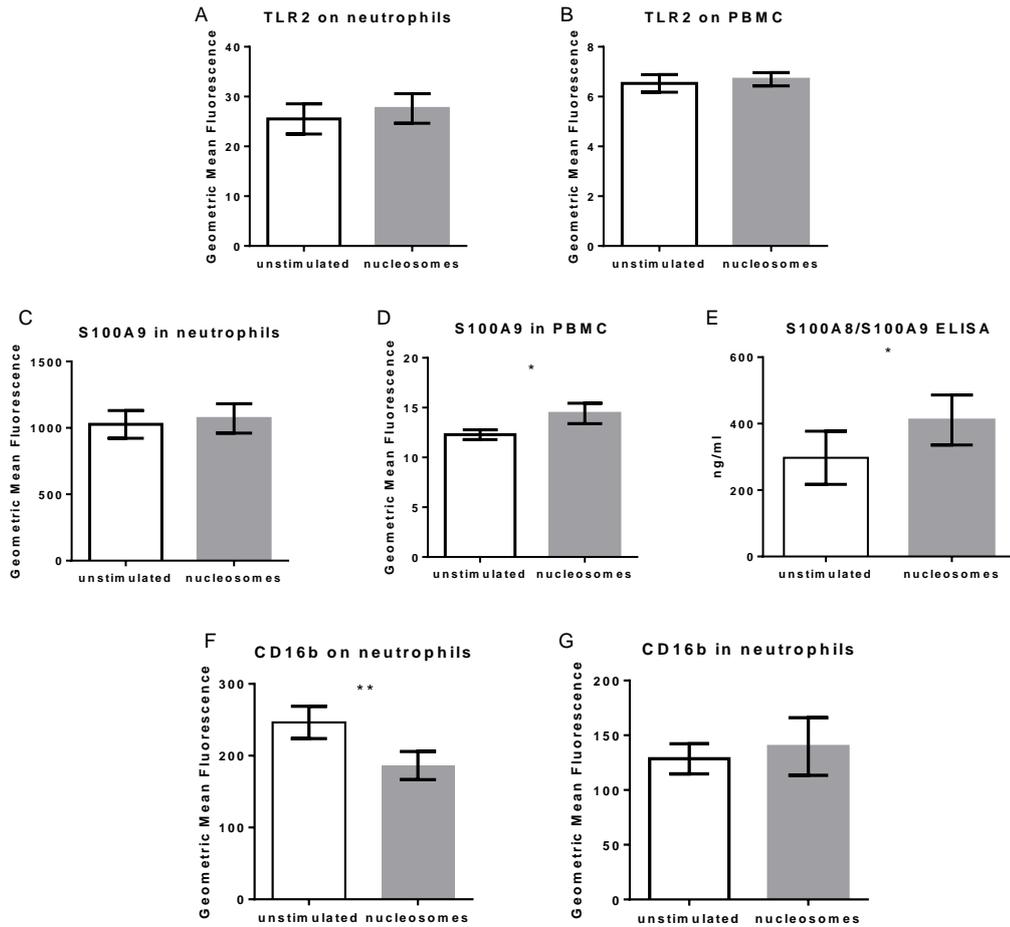


Figure 5.11: Changes in protein levels after 5 h stimulation of whole blood with nucleosomes. Protein expression was measured with flow cytometry and ELISA for eight whole blood samples from healthy adult controls, which were incubated with or without nucleosomes for 5 h. Brefeldin A was added for flow cytometry samples after 1 h. TLR2 expression was unchanged for neutrophils (A) and PBMCs (B). S100A9 expression was also unchanged in neutrophils (C) but was significantly increased in PBMCs (D). Also, the bioactive form S100A8/S100A9 was released significantly after nucleosome stimulation (E). A significant reduction in CD16b was observed in neutrophils (F), but intracellular CD16b (n=7) remained unaltered (G). *p<0.05 **p<0.01 paired t-test or Wilcoxon matched-pairs signed rank test was used depending on results of D’Agostino & Pearson omnibus normality test

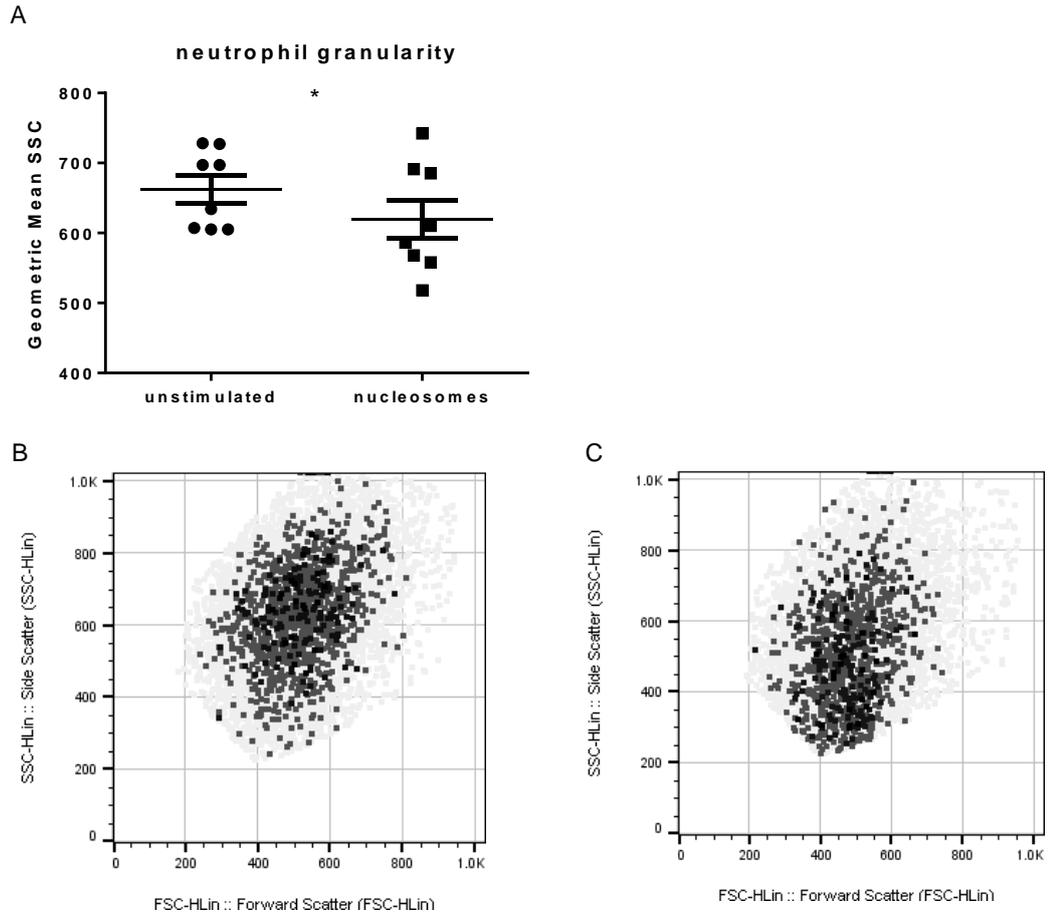


Figure 5.12: Granularity of neutrophils in whole blood stimulated with or without nucleosomes in the presence of Brefeldin A. Granularity was assessed with flow cytometry for eight whole blood samples from healthy adult controls stimulated with or without nucleosomes for 5 h with Brefeldin A being added after 1 h. Differences were observed by measuring the sideward scatter (SSC). A significant decrease in granularity was observed for nucleosome stimulated PMN compared to unstimulated (A). A representative dot plot of one donor's neutrophil population for unstimulated cells can be seen in (B) and for nucleosome treated cells in (C). Drop in SSC is indicating reduced granularity. * $p < 0.05$ with paired t-test after D'Agostino & Pearson omnibus normality test

5.5.2.2 IGS induction by nucleosomes in whole blood

The IGS was also investigated after nucleosome stimulation and was significantly altered (**Figure 5.13**). NETs, in which nucleosomes are present, are considered a cause of IFN α production [256]. To investigate which effects on the IGS are due to IFN α , additional incubations with nucleosomes and 5 μ g/ml anti-IFN α -receptor antibody or Isotype control antibody were performed. Results show that nucleosome stimulation induces IFI44L (p=0.003; **A**), OAS2 (p=0.02; **B**) and LY6E (p=0.006; **C**). Anti-IFN α -receptor antibody significantly reduced the IGS (IFI44L p=0.04; OAS2 p=0.0499; LY6E p=0.006) showing that IFN α causes the IGS resulting from the presence of nucleosomes. This effect was indeed due to a targeted block of the IFN α -receptor, as the isotype control showed significant increase compared to the anti-IFN α -receptor antibody treatments and restore IGS levels as seen for nucleosome only stimulation (IFI44L p=0.04; OAS2 p=0.016; LY6E p=0.022).

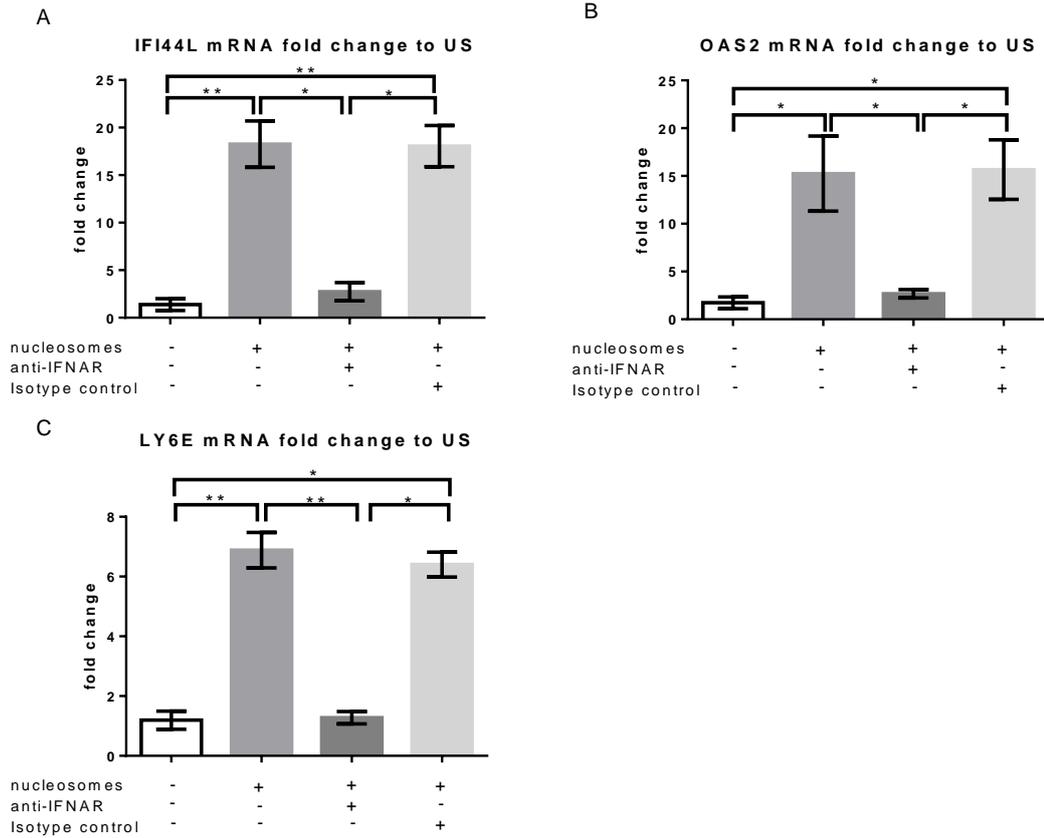


Figure 5.13: IGS after nucleosome stimulation of whole blood for 5 h with or without IFN α -receptor antagonist or Isotype control. Whole blood (n=8) was stimulated for 5 h with nucleosomes with or without 5 μ g/ml anti-IFN α -receptor antibody or Isotype control. mRNA expression was measured with SYBR green using real-time PCR. IFI44L (A), OAS2 (B) and LY6E (C) were measured and results were normalized to the geometric mean of UBC and TOP1. Results are shown as fold change compared to unstimulated whole blood of donor 5. This donor was picked as results from donor 5 represented the average of healthy controls. Nucleosomes induced significant increase of all three IGS. This was significantly reduced with anti-IFN α -receptor antibody, but unaffected by Isotype control. One-way ANOVA with Tukey's multiple comparison or Friedman test with Dunn's multiple comparison after D'Agostino & Pearson omnibus normality test

5.5.3 The effect of cytokines from nucleosomes and apoptotic supernatant stimulation

Both apoptotic supernatant and nucleosome stimulations caused responses in the IGS indicating IFN α involvement. This has further been confirmed as addition of anti-IFN α -receptor antibody reduced this signature (**Figure 5.13**).

Another cytokine discussed in the involvement in JSLE is TNF α , a protein considered pro-inflammatory. TNF α release following nucleosome stimulation was therefore measured in the supernatants (**Figure 5.14**) which were collected as described in 5.4.2. Indeed, nucleosome-treated whole blood supernatants showed a significant increase ($p=0.03$) in TNF α , reaching concentrations over 1 ng/ml.

Results from this work suggest that both cytokines, IFN α and TNF α play an important role in JSLE disease activity. Consequently, their role on neutrophils specifically focusing on the PGS and IGS was studied further.

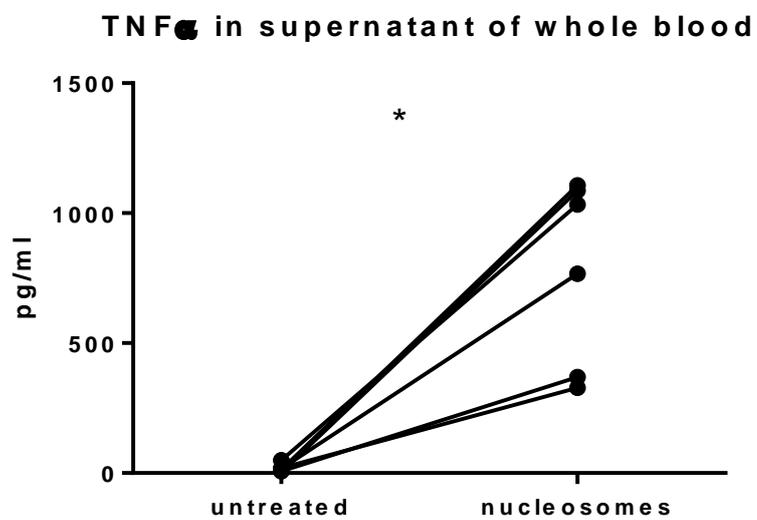


Figure 5.14: TNF α release after 5 h nucleosome stimulation of whole blood. Supernatants obtained from whole blood stimulations (n=6) with or without nucleosomes were measured for release of TNF α with ELISA. Results show significant increase of TNF in the presence of nucleosomes with up to 1.106 ng/ml. * p<0.05 Wilcoxon matched-pairs signed rank test

5.5.3.1 *The influence of IFN α and TNF α on PRGs*

IFN α was used at a concentration of 10 ng/ml and TNF α at 1 ng/ml as this was the maximum concentration resulting from whole blood stimulation with nucleosomes. Neutrophils from paediatric control patients were stimulated for 2 h with IFN α and 30 min with TNF α for mRNA expression and for 7 h (IFN α) and 2 h (TNF α) for protein expression. Neither IFN α nor TNF α stimulated mRNA expression of TLR2 (**Figure 5.15** p=0.16 **A**; p=0.13 **B**) or S100A9 (p=0.56 **C**; p=0.13 **D**). Furthermore, TNF α did not affect Fc γ RIIIb mRNA expression (p=0.19), but it was significantly increased in the presence of IFN α (p=0.03).

The next step was therefore to investigate CD16b expression with IFN α stimulation. As described in 5.4.3, PMN were incubated for 7 h with 10 ng/ml IFN α and 10% FCS. GM-CSF was used as a positive control for CD16b induction. Protein expression was measured with flow cytometry and ELISA (**Figure 5.16**).

GM-CSF significantly induced extracellular expression of CD16b protein (p=0.02) compared to unstimulated PMN and, additionally, a higher intracellular expression was observed (p=0.05; **A**). IFN α had no effect on extracellular levels (>0.99) but increased internal stores significantly (p=0.02; **A**). No shedding into the supernatants was observed for either IFN α or GM-CSF (>0.99 and 0.41 respectively, **B**).

mRNA expression was unaltered after TNF α stimulation for all the PRGs and therefore all proteins were examined (**Figure 5.17**). TLR2 showed significant increase in protein expression for paediatric control patient neutrophils stimulated with TNF α (p=0.03; **A**). While there was no difference for S100A9 (**B**) and CD16b on the cell surface (**C**) (p=0.16, p=0.31, respectively). A trend for decrease in intracellular CD16b levels (**D**) was observed (p=0.09). Results of an ELISA showed no difference in S100A8/S100A9 (**E**) or CD16b (**F**) release (p=0.84 and p=0.88 respectively). This is similar to observations described in 5.5.2.1, where CD16b was decreased in the presence of nucleosomes, although it had only decreased extracellular protein and mRNA.

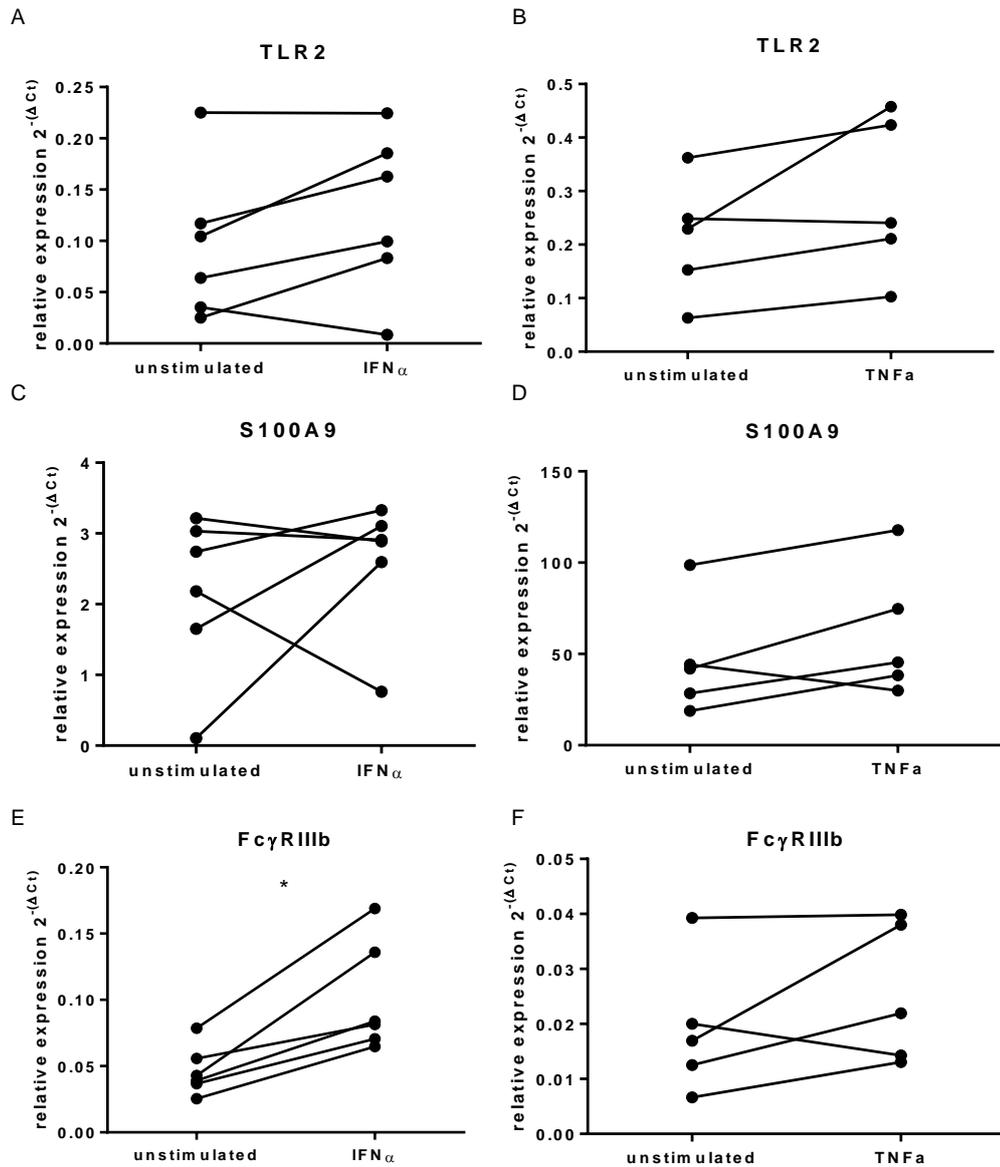


Figure 5.15: mRNA expression of PRGs after stimulation of neutrophils with IFN α and TNF α . Neutrophils of paediatric control patients were stimulated for 2 h with 10ng/ml IFN α (n=6; A, C, E) or 30 min with 1 ng/ml TNF α (n=5; B, D, F). mRNA expression of TLR2 (A, B), S100A9 (C, D) and Fc γ RIIIB (E, F) was measured using real-time PCR with SYBR green. Results are shown as relative expression and PRGs are normalized to ACTB for IFN α and to the geometric mean of ACTB and TOP1 for TNF α . Only Fc γ RIIIB showed a significant increase and only with IFN α . * p<0.05 Wilcoxon matched-pairs signed rank test

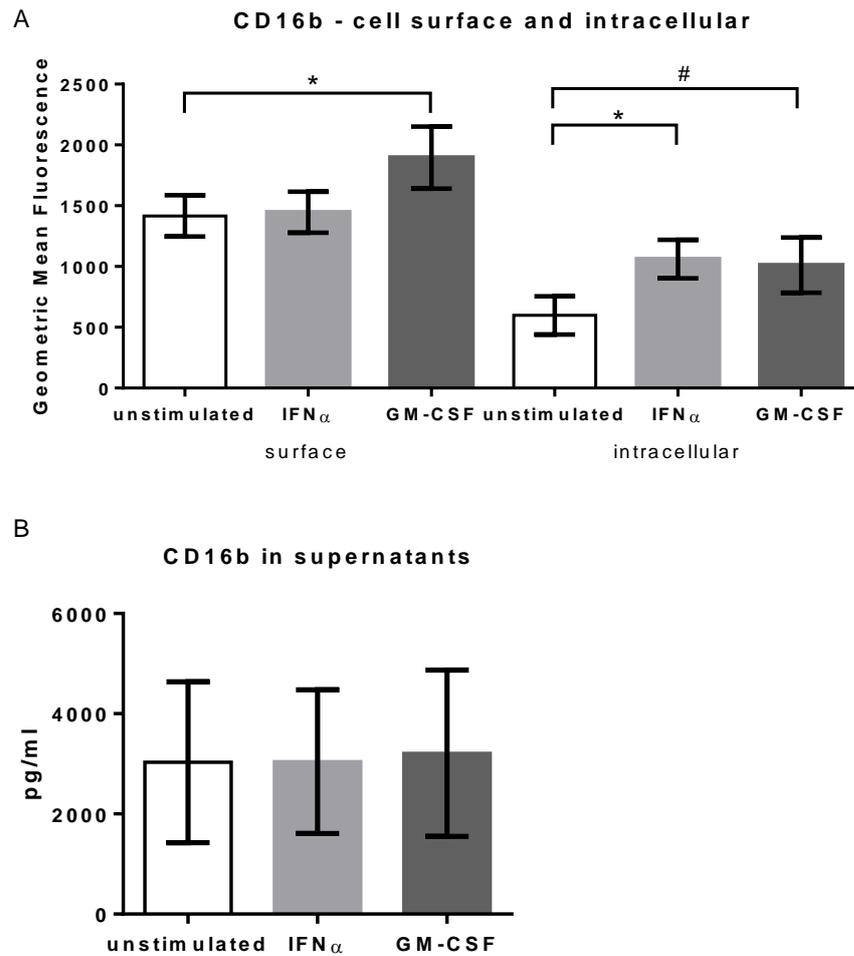


Figure 5.16: Stimulation of paediatric control patients' neutrophils with IFN α or GM-CSF for 7 h. Neutrophils were stimulated with 10 ng/ml IFN α or 5 ng/ml GM-CSF (n=5) for 7 h. Cell surface and intracellular CD16b protein expression was measured with flow cytometry (A). Release of CD16b into supernatants was assessed with ELISA (B). GM-CSF was significantly increased on the cell surface and a higher expression was observed intracellularly. IFN α increased only intracellular stores significantly, but not cell surface expression. # p=0.05, * p<0.05 Friedman test with Dunn's multiple comparison

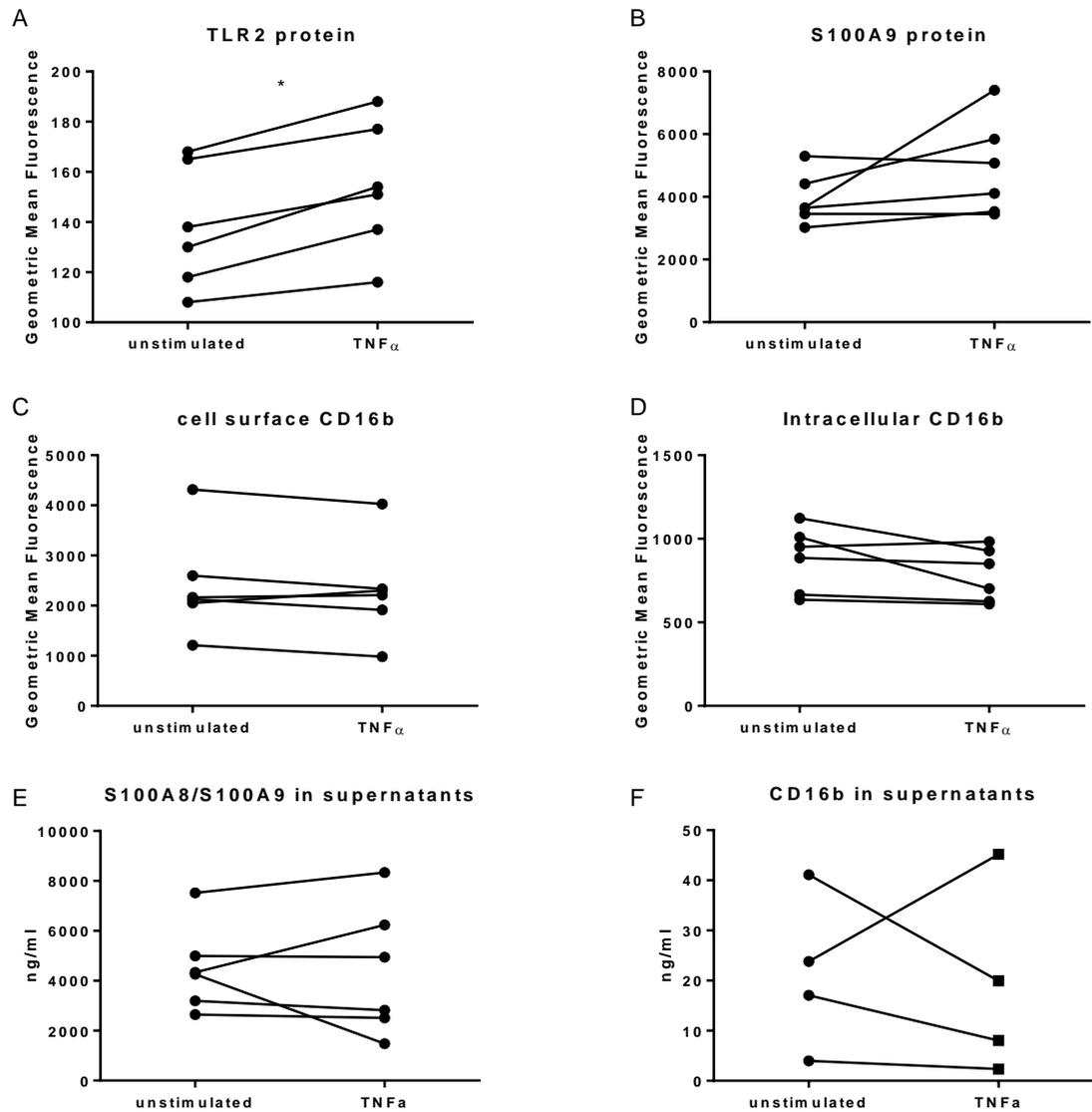


Figure 5.17: Protein expression of PRGs after TNF α stimulation. Paediatric control patient neutrophils were stimulated with 1 ng/ml TNF α for 2 h. Then protein expression was measured with flow cytometry (n=6; A-D) and ELISA (n=5 E, n=4 F). TLR2 protein was significantly elevated with TNF α . S100A9 (B), CD16b on the cell surface (C) and intracellular CD16b (D) was unaltered. Furthermore, S100A8/S100A9 (E) and CD16b (F) were not released into the supernatants. * p<0.05 Wilcoxon matched-pairs signed rank test

5.5.3.2 The influence of IFN α and TNF α on the IGS

Stimulations with nucleosome and apoptotic supernatant can change the IGS and it was therefore important to investigate the influence of IFN α and TNF α on these genes. Neutrophils of paediatric control patients were stimulated for 2 h with 10 ng/ml IFN α or 30 min with 1 ng/ ml TNF α before mRNA expression was measured (**Figure 5.18**).

IFN α stimulation led to a significant increase in OAS2 (p=0.03; **A**) and IFI44L (p=0.03; **C**). LY6E showed higher expression with IFN α but this was not statistically significant (p=0.16; **E**). TNF α stimulation did not cause changes in any of the IFN-induced genes after the 30 min incubation (OAS2 p=0.44 **B**; IFI44L p=0.63 **D**; LY6E p=0.63 **F**).

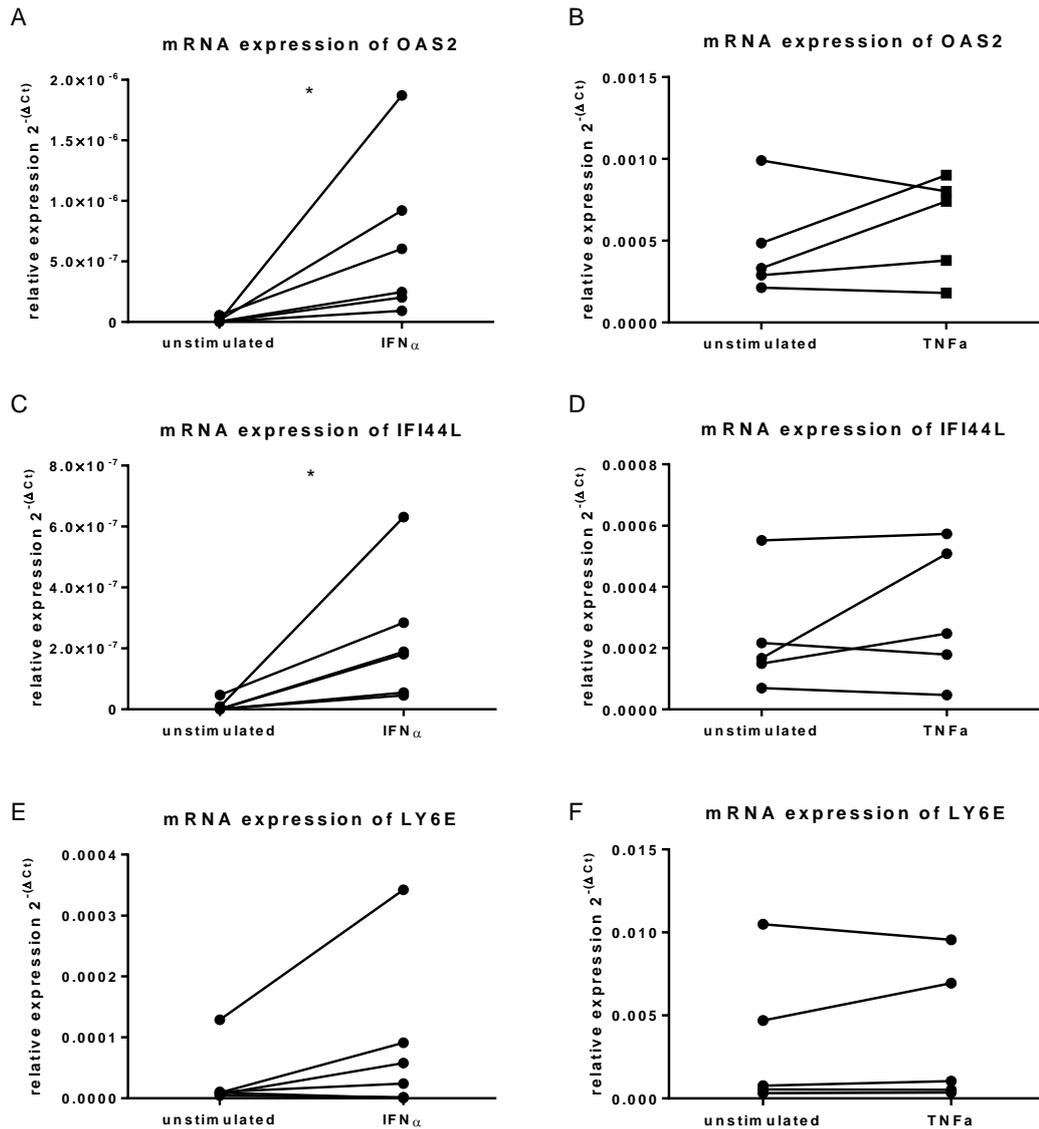


Figure 5.18: mRNA expression of IGS after stimulation with IFN α or TNF α . Neutrophils of paediatric control patients were stimulated for 2 h with 10 ng/ml IFN α (n=6; A, C, E) or 30 min with 1 ng/ml TNF α (n=5; B, D, F). mRNA expression of OAS2 (A, B), IFI44L (C, D) and LY6E (E, F) was measured using real-time PCR with SYBR green. Results are shown as relative expression and IGS are normalized to ACTB for IFN α and to the geometric mean of ACTB and TOP1 for TNF α . OAS2 and IFI44L were significantly increased and a non-significantly higher LY6E expression was measured with IFN α . TNF α did not alter any of the IFN-induced genes. *p<0.05 Wilcoxon matched-pairs signed rank test

5.6 Discussion

In this study the influence of apoptotic supernatant, nucleosomes, IFN α and TNF α on the expression of TLR2, S100A9 and Fc γ RIIIb was assessed. PMN incubated with apoptotic supernatant in the presence of PBMCs and whole blood in the presence of nucleosomes, showed a decrease in Fc γ RIIIb. However, IFN α alone upregulated Fc γ RIIIb expression while TNF α showed no effect. To date, there have been no published studies looking at the neutrophil mRNA expression of Fc γ RIIIb in response to apoptotic supernatant, nucleosomes, TNF α or IFN α .

In strong contrast, TLR2 expression was not altered following stimulation with either apoptotic supernatant, IFN α or nucleosome treatment. TNF α did not change TLR2 mRNA expression of neutrophils after 30 min, but protein expression had increased significantly on the cell surface after 2 h. Previously, in other studies the mRNA of this receptor was found to be upregulated with TNF α in monocytes from healthy adult donors. The authors had incubated the cells for 1.5 h and with 100 ng/ml [257]. In this present study, only a maximum concentration of 1 ng/ml was released by cells from whole blood from healthy adult controls when stimulated with nucleosomes. Furthermore, phorbol 12-myristate 13-acetate (PMA) differentiated THP-1 cells have also been shown to have increased TLR2 mRNA expression after 6 h with 20 ng/ml of TNF α [258]. Therefore, a longer incubation time than 30 min for mRNA expression may have been more appropriate. Zarembek and Godowski also identified IL-6, LPS, Gram-positive and Gram-negative bacteria to be inducers of TLR2 gene expression which we have not investigated in this present study [258].

Similar to the TLR2 increase with TNF α , calprotectin, which contains S100A9, was observed to be increased by TNF α in other studies, for example in human keratinocytes. This effect was only visible after an incubation of 24 h [259]. TNF α incubation in conditions used in the studies presented in this chapter did not show any effect on S100A9 expression and nor did any other stimulation investigated. On the other hand, release of S100A8/S100A9 was significantly increased upon stimulation of healthy adult control whole blood with

nucleosomes. Calprotectin has not been linked to the presence of nucleosomes yet, except for both being present in NETs [260]. Even though an increased release with TNF α was not seen after 2 h with 1 ng/ml, a stimulation with 100 ng/ml of TNF α for 30 min in monocytes has previously shown significant release compared to unstimulated cells. Another explanation for calprotectin secretion may also be due to IL-1 β which has also been shown to cause its release [261], but has been neglected in this study.

Observations in changes of the mRNA in response to factors tested in this study are relevant to neutrophil cell environment of JSLE patients. For example, the increase in Fc γ RIIIb mRNA levels only observed in JSLE patients with an IFN high signature can be explained with observations that IFN α stimulates both the IGS as well as Fc γ RIIIb.

The findings for Fc γ RIIIb mRNA increase after IFN α stimulation was supported with an intracellular increase in CD16b protein expression. There is a lack of studies investigating induction of CD16b in neutrophils, but it has been stated that Fc receptors are significantly increased after stimulation with IFN α on human monocytes and HL-60 cells [262].

In contrast, TNF α incubations and stimulations with nucleosomes showed decrease in CD16b protein expression, but not in its shedding. It has been suggested that this cytokine can induce apoptosis in neutrophils which is expected to decrease extracellular CD16b by shedding [193], [263]. As a result of assay technical issues, we could not measure shedding following stimulations with nucleosomes to confirm that the protein is cleaved into the supernatant due to the presence of TNF α .

While phagocytosis related genes have not been investigated much in the past, there was more work done on the IGS. Nevertheless, despite the importance and strong prevalence of the IFN signature in neutrophils [56], only very few studies have conducted research on the cause of this signature.

IFN-induced genes which make up the IGS in our study were OAS2, IFI44L and LY6E. OAS2 and IFI44L were significantly and LY6E was non-significantly increased with IFN α stimulation after two hours. All three genes have been

shown to be increased in monocytes after a 1.5 h stimulation in whole blood with either IFN α 2a or IFN γ , but high concentrations of 100 ng/ml were used [257]. Interestingly in their study, they also assessed the influence of TNF α and measured decrease of OAS2 and IFI44L after cytokine treatment and no effect on LY6E. OAS2, IFI44L and LY6E was also induced by IFN β in human PBMCs when incubated for 2 h with 100 U/ml [264].

During the stimulation assays with nucleosomes in this present study, the initiation of an IGS was blocked completely in the presence of anti-Interferon- α/β Receptor Chain 2 antibody. IFN γ may have been present but has not induced an IGS. Alternatively, there was no IFN γ release upon nucleosome stimulation as the antibody should not block its action and an IGS should be visible assuming this cytokine can increase the signature. IFN β may have been present and would have been blocked by this antibody.

The stimulation with apoptotic supernatant did not indicate presence of IFN-I in the supernatant itself. Bead-purified neutrophils in the presence of supernatant did not show a strong induction of IGS. In the presence of 5% PBMCs on the other hand, a strong induction of IGS was observed. This may be in response to free nucleosomes released from early necrotic cells or to apoptotic vesicles containing dsDNA. The latter idea is supported by a paper published in June 2018 where the authors found apoptotic vesicles in SLE patients' sera [265]. They contained dsDNA and these vesicles were therefore able to induce an IGS. Most papers had focused on the effect of IFN α on apoptosis rather than the cytokine release due to an apoptotic environment [266], [267].

5.7 Conclusions

In summary, components such as nucleosomes, apoptotic environment, TNF α and IFN α , all part of the neutrophil environment of JSLE can indeed cause changes in expression of PRG or IGS in healthy control neutrophils similar as observed in JSLE patients. IFN α can increase mRNA expression of Fc γ RIIIb in neutrophils. TNF α increased protein expression of TLR2 and IFN α increased

CD16b expression in neutrophils and nucleosomes stimulated protein expression of S100A9 in monocytes as well as release of S100A9. Apoptotic supernatant triggered increased shedding of CD16b which may be part of flares in JSLE, but samples of flaring patients were not available to confirm this hypothesis. All components except for TNF α further caused neutrophils of healthy paediatric controls to show IGS induction as observed in JSLE IFN high patients. For nucleosomes it was further confirmed that they induce IGS via IFN α .

Higher expression of TLR2 and S100A9 as well as low IGS may indicate that IFN low patients have a signature more driven by TNF α , whereas IFN high patients with an IGS and high expression of Fc γ RIIIb are driven by interferons.

6 Discussion and future directions

6.1 Concept of this study

Autoimmune diseases are characterised by autoantibody production by B-cells triggering a mechanistic pathway resulting in disease pathology [2]. Notably, for several autoimmune diseases, the enhanced expression of a genetic signature of neutrophil-related genes has been described, indicating the potential specific importance of neutrophils also in the disease aetiopathogenesis [19], [55]. In SLE, both tissue damage as well as the triggering of disease onset has been associated with neutrophils. Increased and dysregulated neutrophil apoptosis [137] and NETosis in JSLE [256] leads to nuclear auto-antigen presentation to the adaptive immune system and production of proteases that can harm the surrounding tissues [237], [268]. NETosis has been noted to be impaired, probably because of dysregulated phagocytosis. Furthermore, the presence of an increased IGS has been directly linked to increased expression of neutrophil-related gene expression [55].

The overarching hypothesis of this thesis was therefore to determine if it is possible to stratify patients with autoimmune diseases such as JSLE, based on their metabolite profiles in urine and serum and based on differential expression of phagocytic and IFN-induced genetic signatures. In view of their potential for providing a key, important contribution to disease pathogenesis, there was a specific emphasis on the role of neutrophils, and to explore potential inducers of these signatures.

This hypothesis was therefore explored in a stepwise manner. Firstly, the metabolite signatures of JSLE patients and of JIA patients in both serum and urine were analysed and compared to signatures from healthy paediatric control patients. Secondly, the transcriptional signature of neutrophils was explored, including investigation of phagocytosis-related as well as IFN-induced genes. This included translation of phagocytosis-related genes into protein and

functional analysis. Finally, potential causes for these signatures from the JSLE environment were investigated.

6.2 Metabolomics analysis

Investigation of the metabolite signature with ^1H NMR spectroscopy was conducted with the following three objectives for Chapter 3:

- **Objective 1:** To determine if serum or urine is better for building a model to distinguish between metabolite profiles of JIA, JSLE and healthy paediatric control patients, and between the JSLE IFN high and low subtypes and healthy paediatric control patients.

Comparison of PLS-DA results of urine and serum metabolites showed a more informative model created with urine metabolites. Models were characterized by higher robustness and better predictive power between disease and health and between the IFN subtypes of JSLE. These results may have important implications in the future, as urine can be obtained much more easily from patients (especially children and young people) with it being completely non-invasive. Venepuncture can be potentially avoided or at least reduced to a minimum for paediatric patients. However, analysing each metabolite spectrum separately with Chenomx Profiler® is very time consuming and may therefore not be considered as a gold standard. A range of strategies may be adopted to address this limitation. For example, it may be possible to circumvent this problem if a script was developed that normalizes all spectra to the same pH, based on an extensive library which would have to be created with the ppm of metabolites at different pH.

- **Objective 2:** To identify potential pathways differentially regulated between disease groups and healthy paediatric control patients, as well as between the IFN subtypes of JSLE patients.

Combining data from both urine and serum together, four major metabolic pathways were identified to be important for both of the disease processes, JSLE and JIA, namely: Glycolysis, Pentose Phosphate Pathway, Krebs cycle and Urea cycle (summarized Chapter 3, **Figure 3.9**).

Glycolytic activity appeared to be increased for JSLE IFN low patients with products feeding into both the PPP and the Krebs cycle. An upregulated PPP was observed for JIA, JSLE IFN high and JSLE IFN low patients compared to healthy paediatric control patients and has also been described in the literature mainly for T-cells of these diseases (as discussed in 3.5). These data support strongly the importance of the adaptive immunity in disease pathogenesis. On the other hand, both an upregulated PPP and Urea cycle highlight and indicate the importance of neutrophils to this process. PPP shunts increase NADPH production important for phagocytes and ROS production [229]. JIA and JSLE IFN low patients both showed increase in side products from arginine to ornithine conversion, whereas JSLE IFN high patients had higher arginine levels. This seemed not to be converted into ornithine, but potentially directly into citrulline which is an essential protein in NETosis [234]. JIA and JSLE IFN low patients showed increased activity in the Krebs cycle with a higher abundance of inflammatory molecules like fumaric acid or α -ketoglutarate.

Therefore, from a metabolic pathway standpoint, JIA patients appeared to demonstrate greater similarity to that of JSLE IFN low patients in this cohort than paediatric healthy controls, with an emphasis on expression of products reflecting involvement of the adaptive immune system and inflammation. In contrast, JSLE IFN high patients from a metabolic pathway perspective seemed to be directed towards being more NET-producing. This in turn may lead to increased IFN production and later on to autoantibody production. It was surprising to see that JSLE IFN low patients had a metabolic pattern more similar to JIA patients, whereas JSLE IFN high patients were more similar (from a metabolic pathway perspective) to healthy paediatric controls. To date, and in gene expression studies, JSLE IFN high patients have rather been described as being markedly different compared to healthy paediatric controls, and more so than JSLE IFN low patients. In the original study describing the different transcriptomic signatures, JSLE IFN low patients did not demonstrate an increased IFN signature [55]. Sjögren's syndrome patients displaying an increased IGS are considered to have more arthritic and cutaneous clinical manifestations and therefore be more distinct compared to healthy paediatric

controls and IFN low patients with the same disease [247]. It has to be considered however, that medication may play a significant role in this study in the measured metabolite signature. Patients (in this present study) were not all on the exact same medications and this may have influenced results of metabolite changes. JSLE IFN low patients were receiving on average 7.5mg/6.7mg (serum evaluated / urine evaluated patients) of oral prednisolone, whereas JSLE IFN high patients were on an average dose of 5mg/3.6mg (serum evaluated / urine evaluated patients) at the time of sample collection. In metabolite profiles analysed with mass spectrometry, glucose concentration has been shown to positively correlate with prednisolone dose, whereas arginine concentration correlates negatively [269]. In this present study, JSLE IFN low patients were found to be higher in glucose levels and normal in arginine levels compared to JSLE IFN high patients which were normal for glucose, but high for arginine. Contradictory to these findings is that alanine has been previously positively correlated to oral prednisolone [269], but was increased in the JSLE IFN low patient cohort in this present study. These data support the consideration in this study that observations are disease-specific differences in metabolite profiles.

Interestingly, in the same study of Surowiec *et al.*, the main aim was to study fatigue in RA patients. Similar to the present study, they described hypoxanthine, alanine and phenylalanine to be low in RA patients with higher fatigue scores. This may suggest that JSLE IFN low patients and JIA patients are more likely to develop fatigue. They proposed in the paper that xanthine oxidase may be upregulated changing hypoxanthine into xanthine. As a superoxide-producing enzyme this would indicate ROS production. Consequently, ROS production as mentioned above may also cause fatigue. However, to date, no further studies have been identified supporting this hypothesis.

Due to described similarities in their metabolite signatures, it may therefore be worth in the future considering if the disease mechanisms underpinning JSLE IFN low patients may be more driven by Th-17 cells as described in JIA patients [270], whereas JSLE IFN high patients may be driven primarily to produce IFN,

triggered by NETosis. This hypothesis is supported by the fact that SLE patients have been found to possess specific IL-17 producing T-cells [131]. The study by Crispín *et al* did not present any details about each single patient, but another study looking at JSLE patients did [133]. Plasma levels of IL-17 were significantly higher in the JSLE cohort compared to the healthy paediatric control cohort. In this paediatric study, they further stimulated JSLE and healthy control PBMCs with CD3/CD28 and found after two days incubation, a significant increase in IL-17A and IL-17F concentrations in supernatants. Interestingly, only a very small minority of patients reacted with high IL-17 production [133]. Even though in that study they did not look specifically at IFN signature subtype stratification, these very small numbers of JSLE patients' PBMCs reacting with IL-17 production may reflect the JSLE IFN low patient subset. This line of study would warrant further investigation.

6.3 Phagocytosis related profile of patients with JSLE

In Chapter 4, the focus was on the transcriptional signature in JSLE, to confirm and find further evidence for the importance of neutrophils in disease pathogenesis, and to identify if there were additional differences between JSLE IFN high and IFN low patients respectively. This was explored through a series of steps and specific objectives.

- **Objective 1:** To determine an appropriate IGS to differentiate between JSLE IFN low and JSLE IFN high patients.

Initially, four genes were chosen to investigate the IGS, namely IFI44L, OAS2, LY6E and IFI6. However, IFI6 was observed to not correlate with any of the other three genes and was in one patient higher in the PBMC fraction than in the neutrophil fraction. For this selected gene signature in neutrophils, in JSLE IFN low patients it remained low over time. In contrast, for JSLE IFN high patients it remained high, but showed variation in the amount of their gene expression. This may indicate an inherent signature in patients rather than dependence on disease development. Authors of other published data have suggested a potential relationship between IFN signature expression and production of both

BAFF and anti-dsDNA antibodies [220]. However, within the present study and available patient cohort, this could not be confirmed as BAFF was not tested and only one JSLE IFN low patient had developed anti-dsDNA antibodies over time. Consistency over time for a patient to be expressing an IFN low or IFN high signature would indicate potential subgroups of the SLE disease spectrum. Identification of the respective IFN subgroup of a patient (IFN high or IFN low signature expressions) may be a valuable tool to distinguish different treatment responses dependent on their respective IGS. It has been suggested that IFN high patients would respond better to type I IFN receptor antagonist treatment [271]. In the study described, both IFN high and IFN low patients had their own placebo group. The main differences observed was a higher response to treatment in the placebo group of the IFN low patients compared to the placebo group of IFN high patients. This meant that for the IFN low patients treated with the antagonist, there was not a significant difference in their response to treatment. The response rate to the type I IFN receptor antagonist itself was comparable between SLE IFN high and SLE IFN low patients [271]. It has also been reported that IFN low patients respond better to treatment with anti-IFN α than IFN high patients [63]. This may indicate that IFN low patients express and produce interferon protein, but do not respond to it in the same way with an increased IGS, or that a different set of genes has to be used to distinguish between IFN high and IFN low patients for these differences to be shown. The selection of genes that together are used to define an IGS is further discussed in Section 6.5. Of note however, it does need to be considered that in the study of Kulunian *et al.* the genes HERC5, EPST1 and CMPK2 have been used to determine the IGS, but at the same time different genes have been used including OAS2 to determine the effect of the anti-IFN α treatment [63]. As introduced in Section 1.4.2, it is essential to keep in mind that there are many genes related to and that can form part of an IGS. Different approaches using different sets of genes to define an IGS may result in altered outcomes. It may on the other hand just be a reflection that IFN patients are more likely to get better even with placebo treatment, as seen in the study of Furie *et al.* (2017) [271].

Objective 2: To measure the expression of genes that are involved in the modification of DNA and phagocytosis-related genes (PRGs) in neutrophils of JSLE patients with their respective IFN subtypes and healthy controls.

Objective 3: To compare protein expression of PRGs in JSLE neutrophils, and their respective IFN subtypes, and healthy paediatric control patient neutrophils.

Besides the described increased IGS expression, there was an increase expression observed for genes that are involved in the modification of DNA. However, there was an even stronger difference for the increased PGS noted in neutrophils of JSLE patients consisting of TLR2, S100A9 and Fc γ RIIIb/CD16b. While the increased IGS was only present in JSLE IFN high patients, JSLE IFN low patients also showed in part an increased PGS, specifically for TLR2 and S100A9. Furthermore, the increased PGS was also present at the protein level. However, increased S100A8/S100A9 protein expression may be more related to increased disease activity than to IFN low or IFN high status. TLR2 protein was highest in JSLE IFN low patients which had higher disease activity, in which there may be a direct correlation. This correlation was not further investigated due to the small sample size. However, it has been described that TLR2 activation can increase glycolysis which was also found (see Chapter 3) to be increased in JSLE IFN low patients [272]. Pyruvate, which is part of the glycolysis pathway, was detected in urine metabolite analyses to be increased in JSLE IFN low patients compared to JSLE IFN high patients. However, there was no difference in the SLEDAI-scores (average of 9.3 for IFN low / 9.8 for IFN high patients). CD16b expression was more dependent on IFN status and therefore, CD16b, more specifically serum and intracellular CD16b, could be identifiers for IFN high patients. It would require further investigation to validate this finding.

Intracellular S100A9 protein was increased within neutrophils of JSLE patients, independent of IGS stratification. S100A9 protein expression inside neutrophils may therefore be a candidate protein helping identify JSLE patients, or may simply be a marker for autoimmune diseases in which there is an importance of neutrophil involvement. Nevertheless, importantly, for both JSLE and JIA patients, S100A9 levels within neutrophils have not previously been reported. It

has to be noted, that S100A9 is important in the disease both alone as S100A9 and as calprotectin in the S100A8/S100A9. For example, in serum or plasma S100A8/S100A9 has been used as a biomarker for disease activity in sJIA [273]. In arthritic mice S100A8 and S100A9 have been detected in damaged joints and the calprotectin levels in sera of these mice reflected cartilage damage and bone erosion. It has also been demonstrated in an arthritis mouse model, that anti-S100A9 reduces arthritis and inhibits release of pro-inflammatory cytokines. Calprotectin is strongly upregulated in sera of these arthritic mice if they were not treated with anti-S100A9 [274]. Nonetheless, the specific increased expression of intracellular S100A9 within neutrophils has not been previously investigated in JIA or JSLE.

- **Objective 3:** To investigate the phagocytic ability of JSLE neutrophils compared to healthy control neutrophils.

The PGS of JSLE patients indicated increased phagocytic activity which was confirmed with short incubations of 20 min with pHrodo coated bioparticles which included *S.aureus*, *E.coli* and zymosan. Neutrophils of healthy paediatric controls and JSLE patients were investigated together with serum of healthy paediatric control patients and showed increased phagocytosis by JSLE neutrophils compared to ones of healthy paediatric control patients. Unfortunately, it was not possible to undertake further differential investigation of phagocytosis in JSLE IFN high and IFN low patients respectively, for a number of reasons. Firstly, this was due to not enough JSLE IFN low patients being available during the period studied and secondly, because there was no clear separation between IFN low and IFN high patients in the number of phagocytosed particles identified. Thirdly, the main difference between JSLE IFN high and IFN low signatures was in CD16b expression, which is responsible for opsonized particles. It was therefore not expected to have a direct effect in this assay.

An important finding from these data identified that JSLE neutrophils were observed to be in an activated state, enabling them to respond quicker to phagocytic stimuli. Other studies had suggested that after longer incubations with bacteria in the presence of JSLE serum, less phagocytosis was observed in

patients compared to controls (as discussed in 4.6). This may indicate that there are also factors in the serum rather than an intrinsic issue in neutrophils, which potentially inhibit phagocytosis and may trigger hyperactive neutrophils to undergo NETosis rather than phagocytosis. If this is the case, the data presented in this present study indicates that this would be independent of the IFN signature observed in patients, and furthermore, could be a contributing factor in the underlying disease mechanism for JSLE patients in general.

Another factor potentially contributing to the serum effects are treatment that patients receive. Both cyclophosphamide and prednisolone are considered inhibitors of phagocytosis, therefore the incubation without serum and with healthy control serum gives specific insight into neutrophil rather than medication specific activity [275], [276]. Nonetheless, it is worth mentioning that none of the patients who donated blood for the phagocytosis assays was on cyclophosphamide and four out of five patients were on prednisolone. Therefore higher phagocytic activity is an even more striking finding.

6.4 The influence of the environment of JSLE neutrophils

The aim of Chapter 5 was to investigate potential causes of the increased IGS and PGS observed in JSLE patients. Potential influences from the neutrophil environment in JSLE patients were tested on their influence on the PGS and IGS. These included apoptotic supernatant of healthy control neutrophils, nucleosomes, IFN α and TNF α .

The main objectives were:

Objective 1: To investigate the impact of the apoptotic environment, nucleosomes, IFN α and TNF α on gene and protein expression of PRGs in neutrophils or whole blood.

Fc γ RIIIb mRNA expression decreased in the presence of apoptotic supernatant of neutrophils when PBMCs were present. At the same time, this stimulation triggered the shedding of CD16b protein into the supernatant. Fc γ RIIIb/CD16b mRNA as well as protein expression was decreased with nucleosome stimulations of whole blood but increased with IFN α . These results would

suggest that JSLE IFN high patients have IFN α present and increased cytokines from apoptotic supernatant. Nucleosomes induced production of IFN α in whole blood, but additionally release of up to 1 ng/ml TNF α . The combination may have inhibited the upregulation of CD16b. Nucleosomes without the presence of other cytokines from the apoptotic environment could partly explain CD16b low neutrophil signatures in IFN low patients.

TNF α as seen in nucleosome stimulations of whole blood increased TLR2 protein expression on neutrophils. TLR2 could be high in IFN low patients because of the presence of TNF α . This cytokine also plays a role in JIA [277] and has been proposed to be important for lupus as discussed in Section 1.6.1.2. The results of this study showed that TNF α is released upon nucleosome stimulation and that TLR2, a protein increased by TNF α , is increased on JSLE patients' neutrophils. These data all support the findings of Chapter 3 that JSLE IFN low patients are more similar (from a metabolic perspective) to JIA patients.

The final protein making up the PGS was S100A9 which was observed to have increased protein expression in PBMCs after stimulation with nucleosomes in whole blood. Expression was unaffected by any other treatment as well as in neutrophils. Nucleosome stimulation triggered release of the bioactive form S100A8/S100A9 the source of which may be neutrophils. Despite unchanged intracellular protein levels this stimulation may have caused increase of calprotectin. Measurement with flow cytometry detected decreased granularity after encounter of nucleosomes. Unchanged levels of S100A9 could therefore indicate release of preformed granules and replenishment of intracellular stores. S100A8/S100A9 has been described to be increased in disease active patients [253] and encounter of nucleosomes may therefore be a trigger of flares. This coincides with a study reporting nitrated nucleosomes in 63% of all patients and serum levels were associated significantly with vasculitis flares [278].

Objective 2: To measure the influence of the apoptotic environment, nucleosomes, IFN α and TNF α on gene expression of IGS in neutrophils or whole blood.

The IGS measured in neutrophils was increased by stimulation with IFN α . Furthermore, apoptotic supernatant increased the IGS, but only when PBCMs were present which suggests PBCMs release IFN α . Nucleosome stimulation of whole blood increased the IGS, too, which was due to IFN-type I. This was demonstrated by blocking of IFNAR which normalized the expression of IFN-induced genes whereas isotype control stimulations with nucleosomes showed high IGS. Nucleosomes may be present in apoptotic supernatant causing the IGS to rise. Centrifugation of apoptotic supernatant was conducted only below 113.000xg, the speed necessary to sediment nucleosomes. Summarising the results from Chapter 5, the strongest effect on the IGS was seen with stimulation of IFN α alone. This cytokine can be stimulated by both supernatant of dying, uncleared cells or purified nucleosomes.

While the IGS in this study only comprised JSLE IFN low and IFN high patients, there are also studies, for example in adult-onset SLE, describing several disease phenotype subtypes which includes differential expression of IFN as well as of TNF α . These subgroups include: IFN low/TNF low, IFN high/TNF low, IFN high/TNF high and IFN low/TNF high [279]. These data support the hypothesis resulting from findings of Chapter 3-5, that JSLE patients have a signature potentially depending on both IFN α and TNF α . This hypothesis is supported by the data in this present study indicating that JSLE IFN low patients may be more similar to JIA patients in disease aetiopathogenesis as they show similarities in their metabolite and cytokine profiles. As described in Section 1.6.1.2, where the role of TNF α in SLE has been outlined, both the absence and presence of TNF α has been assumed to play a role in SLE disease pathogenesis [126]–[128]. The occurrence of patients in different subgroups of expression of TNF α as TNF α high and TNF α low patients may explain controversial findings that both the absence and presence of TNF α can induce SLE. IFN α expression and IFN-inducible genes have been shown to increase upon blockade of TNF α with etanercept in healthy PBMCs *in vitro* [280]. Addition of TNF α to these cultures with etanercept reversed the effect and reduced expression of IFN α and its inducible genes [280]. Presence of TNF α in JSLE IFN low patients could therefore explain the absence of an increased IGS. Pre-incubation with IFN α on

the other hand can inhibit TNF α production of macrophages stimulated via TLR2, TLR4 or Fc γ R [281]. A disproportion of either TNF α or IFN α may suppress the respective other cytokine leading to even stronger imbalance and resulting in IFN low or IFN high JSLE patients.

6.5 Future directions

Urine has been identified in this study as an accessible and useful biofluid to help establish a model of distinguishing metabolite profiles of patients with autoimmune diseases from healthy paediatric patients. However, it has also been shown to be quite a laborious process with the associated analysis, which may prevent its future development, validation and potential implementation into routine use. A study with an increased number of JIA and JSLE patients including investigating the expression of different IFN subtypes would first need to confirm the observed separations within the urine and serum models. These would then need to be validated in independent cohorts. From here, the metabolites most differentially expressed between the groups, and at the same time most influential on the PLS-DA model, would need to be determined. This would then allow samples to be compared solely for peaks relating to these specific metabolites, which would drastically reduce analysis time.

Metabolomic pathway analysis revealed that JSLE IFN low patients were more similar to JIA patients than JSLE IFN high patients. One reason could be, that most of the JIA patients are IFN low, as described in Section 3.5. A comparison of metabolite profiles of more IFN low patients from JSLE and JIA patients could help distinguish if the similarity in metabolite signature found in this thesis is IFN low signature specific or may have been related to disease activity for example.

Besides the metabolite signature in urine and serum, the IGS was also investigated in the JSLE cohort. It was found that of the four selected genes, only OAS2, IFI44L and LY6E could be correlated to each other, whereas it was not possible to correlate IFI6 to any of the three other IFN-induced genes. In this study, one example of an IGS has been investigated. However, there may be

different IGS with different implications. Initially, IFI44L, OAS2, LY6E and IFI6 were used in this study as part of the IGS (IFI6 excluded after further analysis). Each of the four genes had been part of an IGS in other studies, but not in combination [57], [63], [64], [68]. While Rice *et al.* included IFI27, IFIT1, ISG15, RSAD2 and SIGLEC1 besides IFI44L, Kalunian *et al.* measured changes in IGS with IFI27, IFI44, IFIT1, MX1, OAS1 and OAS3 alongside OAS2. Other than LY6E Feng *et al.* initially tested expression of OAS1, OAS2, OASL, IFIT1, IFIT4, IFI44, STAT1, ISG15, MX1, MX2, PLSCR1, XIAPaf1, and IRF7 as part of an IGS, but found, that several of those were dependent on each other. They subsequently reduced their IGS to only LY6E, OAS1, OASL, MX1 and ISG15. In their paper they also showed that there are strong differences between the diseases, but they did not test if the genes correlated within one disease [68]. El-Sherbiny *et al.*'s IGS contained IFI6 and IFI44L, together with ISG15, IFI44, IFI27, CXCL10, RSAD2, IFIT, CCL8, XAF1, GBP1, IRF7, CEACAM1, HERC5, EIF2AK2 and MX1. This study focused on the analysis of samples of different diseases, namely RA, SLE and undifferentiated connective tissue disease (UCTD) [64]. Each of these diseases may have a different IGS and be dependent on various factors including several different cytokines. The IGS cluster chosen may therefore be reflective of the disease with most samples, which was UCTD. Correlations studies of IFN-induced genes for a big group of patients may help elucidate influence of genes on each other and can help find disease specific IGS. This approach should also include other signatures, like the PGS and TNF α , to really define patient subgroups with potentially different underlying disease mechanisms.

Besides the importance of the IGS noted in this present study, a PGS has also been observed to be important, with increased phagocytic activity in JSLE patients compared to healthy paediatric control patients. A future study should look at healthy paediatric and JSLE neutrophils incubated with bacteria and JSLE serum. JSLE serum may inhibit phagocytosis leading to NETosis instead. This may be further increased in neutrophils of JSLE patients due to their increased activity to phagocytose seen in the presence of serum of healthy paediatric control patients. This could explain disease development and should therefore be investigated further.

6.6 Limitations

JSLE being a rare disease and IFN low patients being uncommon in JSLE led to low sample numbers for all assays and no possibility to age and sex-match patients. Therefore, all results have to be treated with caution and statistical significance may not have been reached despite a biological difference. Furthermore, while it was possible to retrieve information about disease activity scores for most patients, any missing information may result in missed links between measured markers and disease activity.

Additionally, analysis of metabolite profiles of serum may not show the full picture of disease and health. Metabolites can have several peaks and observed separation can be biased by one metabolite causing a high percentage of the separation as all peaks would change simultaneously. Furthermore, statistical analysis is very strict due to the high number of peaks resulting from good assignment and annotation of metabolites. In this thesis, results of non-adjusted p-values were considered sufficient for further analysis. Nevertheless, this may also cause false-positive results. Similarly, no annotation of peaks leads to loss of data and informative differences may therefore not have been observed in this study. Peaks annotated by Chenomx Profiler® as Ethanol for example were excluded as discussed in Section 3.3.2, but it still remains an option that there is additional information in this data. Ethanol could be produced by the gut microbiota [282] and thereby affect serum levels and health of patients.

Furthermore, in the metabolomics studies, JIA patients were included in the analysis, but no data on their IFN subtypes was available. It was assumed that they were mostly IFN low patients as described [57]. It can therefore not be distinguished if similarity between JSLE IFN low and JIA patients was due to the IFN signatures or to causes in disease. Nevertheless, findings that upregulation of TLR2 as seen in IFN low patients could be caused by TNF α (Chapter 5) supports the hypothesis that factors are in the cell environment of JIA and JSLE IFN low patients are comparable.

Nucleosome stimulations of whole blood showed that $\text{TNF}\alpha$ was released and can play a role in JSLE. Subsequently, only stimulations of $\text{TNF}\alpha$ and $\text{IFN}\alpha$ alone have been studied, but a combination of both may show a profile more similar to JSLE IFN low patients. As described in [283], cytokines can act synergistically and can cause changes that are only observed when both cytokines are present. Moreover, only effects of $\text{IFN}\alpha$ were studied, but yet other type-I IFNs or type-II or -III IFNs may also play a role and influence the IGS and PGS. TLR2 for example was measured to be increased in keratinocytes after a stimulation with $\text{IFN}\gamma$, the type-II IFN [284]. Furthermore, even though altered pathways have not been investigated, stimulation of murine bone marrow derived macrophages with $\text{IFN}\gamma$ resulted in increased phagocytosis of latex particles [285].

Nucleosomes were used for stimulations resulting in differences for the IGS and PGS, but in diseases nucleosomes are often modified upon release. For NETosis neutrophils alter their histones with peptidylarginine deiminase 4 (PAD4) which converts arginine into citrulline and thereby decondensates the chromatin [286]. Anti-citrulline antibodies have been found in RA sera which suggested that these modified proteins are stimuli for the immune system [287]. Results may differ strongly with different types of nucleosomes, although IGS induction should remain high as this is a natural response to dsDNA. Especially evaluation of PGS upon stimulation with modified nucleosomes would be valuable.

The functional effects of the PGS have been studied with pHrodo coated particles and increased phagocytosis was found. However, uptake of live bacteria may give different results and bacterial killing also remains to be evaluated.

The main studied cell type were neutrophils and several dysregulations and differences to healthy paediatric patients were found. Nevertheless, also LDGs were mentioned to play a role in JSLE. Metabolite profiles described in Chapter 3 were created from serum and urine and metabolites of LDGs can contribute to these profiles. However, very few metabolomic studies look at specific cell

subsets [226] and no study has been found that looks at LDGs. This entire subset of neutrophils was not addressed in the investigations outlined in Chapters 4 and 5. Gene expression, protein expression and functional assays investigated in Chapter 4 were limited to normal density granulocytes as cells were obtained by Histopaque separation. LDGs are more likely to undergo NETosis and are considered to be in a more activated state than normal density granulocytes [237]. While in this thesis neutrophils were found to be strongly activated to phagocytose, LDGs have been described to be impaired in phagocytosis which may influence the likeliness of NETosis [145]. A cause for the development of LDGs is not known and they are less frequent in healthy controls compared to JSLE patients [159]. In Chapter 5, blood from healthy controls was used and LDGs were therefore not further investigated.

6.7 Implications of findings

Despite low numbers of samples from patients, comparison of metabolite profiles from urine was superior to comparison of metabolite profiles from serum in distinguishing between diseases and their subgroups. As mentioned in Section 1.5.3, urine is commonly used for distinguishing between active and inactive and the different stages of nephritis. The results from this thesis support the position that to determine metabolite profiles of urine (instead of serum) may also be a tool for investigation of disease pathogenesis, stratification of patients and potentially biomarker discovery for JIA and JSLE.

In this study another novel finding was the increased protein expression of S100A9 within neutrophils of JSLE patients compared to healthy paediatric controls. Even though this may help to distinguish JSLE patients from healthy children and potentially (but not investigated here) from other diseases, intracellular protein expression of neutrophils is not a test which could be performed routinely as a diagnostic test. Nevertheless, decreasing S100A9 production in JSLE could be a therapeutic target and prevent flares as increased release has been associated with higher disease activity as discussed in Section 6.4.

Lastly, JSLE IFN high patients showed to be distinctly different compared to JSLE IFN low patients and healthy paediatric controls in their metabolite profile, in their IGS and their PGS. Consequently, for studies investigating JSLE and its treatment options, and not solely for anti-IFN α treatment, patients should be stratified into subgroups when a study is being set up and when results are being analysed.

6.8 Final conclusions

This thesis presents evidence that specific neutrophil signatures are present in patients with autoimmune diseases like JIA and JSLE. Metabolite profiles of serum and urine show pathways like the PPP to be upregulated which support neutrophil activity especially phagocytosis, and the urea cycle indicating increased NETosis. The IFN-induced gene signature measured in the cohort of JSLE patients in this study has been found not to change over time, if a strict selection of genes is used which includes only the correlating ones, namely IFI44L, OAS2 and LY6E. For the first time JSLE patients were shown to have a phagocytosis-related gene signature which comprised of TLR2, S100A9 and Fc γ RIIIb/CD16b. Differences of Fc γ RIIIb/CD16b were only found in JSLE IFN high patients compared to IFN low and healthy paediatric control patients. Importantly, the PGS was translated into protein and functional assays revealed JSLE neutrophils to be enhanced in phagocytosis. This was a novel finding, as functional assays with JSLE serum and/or long incubation times have given the impression that neutrophils were impaired in phagocytosis. In this study apoptotic supernatant and nucleosome stimulation, both present in the JSLE environment, displayed effects on neutrophils causing both the IGS and PGS to become more similar to the JSLE signatures than to the one in healthy paediatric children. The two main cytokines found to play a role were IFN α and TNF α .

Interestingly, findings within this thesis suggest that JSLE IFN low patients have more in common with JIA patients than JSLE IFN high patients do, for example their metabolite profiles. High TLR2 in JSLE IFN low patients may further

suggest that JIA and JSLE IFN low patients have a similar involvement of cells and cytokine. This hypothesis remains to be tested further.

To conclude, this study has demonstrated that it is possible to stratify JSLE patients by their metabolite profiles in serum, urine and by both phagocytic and IFN-induced gene signatures present in neutrophils. Furthermore, it has shown that the PGS is visible in the phagocytic activity of PMN of JSLE patients and variation of signature expression are caused in part at least by variations in the environment in which PMNs find themselves.

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Appendix A: Research Ethics Approval

Appendix 1: JSLE patient and paediatric control sample ethics



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09 February 2016

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Dear Professor Beresford

Study title:	UK Juvenile Systemic Lupus Erythematosus Cohort Study & Repository: "Clinical characteristics and immunopathology of juvenile-onset systemic lupus erythematosus"
REC reference:	06/Q1502/77
EudraCT number:	N/A
Amendment number:	Substantial Amendment 4
Amendment date:	01 January 2016
IRAS project ID:	

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

Approval was sought for updates made to the protocol.

The Sub-Committee commented that page 11 of the protocol stated that you are proposing to include healthy controls, aged 13-15 from friends and siblings and they requested further information on how these controls would be approached, who will approach them, etc.

You replied that you have provided more detailed information on how you plan to approach these healthy controls in the protocol on page 11 with the following text:

"Visitors aged 13 – 15 years attending the hospital may also be approached where appropriate for example through asking patients to identify friends/siblings who may be willing to take part in the study. Visitors will only be approached in an appropriate, confidential, quiet environment by GCP trained staff on the delegation log. Staff will only approach potential participants where they feel comfortable that it is appropriate timing and the family may be interested. They will provide an appropriate patient information leaflet and offer time to take the information leaflet away and read it in their own time. They will be clear that the family may say no. They will typically be approached by a doctor or nurse during a

A Research Ethics Committee established by the Health Research Authority

clinic setting when the family are attending for another reason, for example, asking patients / parents if they have siblings / other children who may be interested in taking part in the study."

The Sub-Committee also sought further information on where the adult controls would come from, how and who will be approaching them.

You replied that more detailed information on how you plan to approach adult healthy controls in the protocol on page 11 with the following text:

"Adults aged sixteen years or over, without any significant past medical history of inflammatory disease and freely able to consent will be recruited to assist with experiment optimisation at AHCH. Persons working in the Trust or the University (e.g. medical students / administration staff etc.), will be approached and provided with an information sheet. Staff will be approached by GCP trained staff who are familiar with the study and on the study delegation log. They will only be approached in an appropriate, confidential and quiet environment and provided with an information sheet. They will be offered time to take the information leaflet away and read it in their own time. Staff will be clear that they can say no and there is no requirement for them to take part in the study. Only staff that are willing to take part in the study will be asked to sign the consent form."

The Sub-Committee commented that on page 14 of the protocol it stated that the only identifiable details to be stored electronically are email address; the Sub-Committee sought further clarification as to why you can't store these separately and just access them when required.

You replied that you agree that email addresses can be stored separately and accessed when required. You have removed this from the protocol on page 14.

The Sub-Committee asked for clarification on how much blood is to be taken, for example, will it be 5ml/1 teaspoon. They requested that the PILs are changed to state the amount of blood to be taken.

You replied that the volume of blood to be taken is 2-3 teaspoons. You have revised the PILs and resubmitted them.

The Sub-Committee requested that in the section "Do I have to help" in the PILs, it should start with "no". They noted that some of the PILs already do this, but not all of them.

You updated all the PILs and resubmitted.

In the PILs for the inflammatory controls for <6 and 6-12, the Sub-Committee noted that it does not state that participants have been chosen because they have arthritis, but does in the PILs for 13-15 year olds and sought further clarification as to why this is the case.

You updated the PILs accordingly and resubmitted.

The Sub-Committee commented that the PILs do not mention being flagged with the HSCIC, or informing the GP and sought further clarification for this.

You replied that you are only using HSCIC flagging on JSLE recruits and this was already included on the PILs for the 13-15 year olds, >16 year olds and parents. You said that you would not be using the HSCIC service for any of the control recruits. You also said that informing the GP was already included in all the PILs except for the adult healthy controls, which you amended and resubmitted.

A Research Ethics Committee established by the Health Research Authority

The Sub-Committee commented that there was no contact information provided for complaints in the PILs.

You replied that you have updated the PILs to include the following in the parent and participant >16 years PILs:

"What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help. If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team."

The Sub-Committee sought further clarification as to whether participants can still take part if they do not agree to flagging with the HSCIC and/or if they do not want DNA analysis.

You replied that patients can still be involved in the study if they don't agree to flagging with the HSCIC and/or if they don't want DNA analysis by not initialling the appropriate boxes on the consent form. Each line of the consent form needs to be initialled specifically, and any not included such as these are noted. You said that to date, none have not wanted to take part in all of the components, but it remains always an option. You also mentioned that this is already included in the protocol for HSCIC on page 16.

You went to say that you have updated the protocol regarding DNA analysis on page 18 with the following sentence:

"Participation in this section of the study requires the patient to specifically initial a box saying 'I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else'. Patients who do not want their DNA to be analysed can still participate in the rest of the study."

You went on further to state that you have included an additional point on the assent/consent forms regarding DNA analysis:

"I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else."

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Covering letter on headed paper		29 December 2015
Notice of Substantial Amendment (non-CTIMP)	Substantial Amendment 4	01 January 2016
Other [Response to Committee Queries]		05 February 2016
Participant consent form	4	01 January 2016
Participant consent form [Controls]	4	01 January 2016

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Participant information sheet (PIS) [Controls]	4	01 January 2016
Participant information sheet (PIS)	4	01 January 2016
Research protocol or project proposal	4	01 January 2016

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

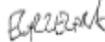
Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at <http://www.hra.nhs.uk/hra-training/>

06/Q1502/77:	Please quote this number on all correspondence
--------------	--

Yours sincerely



On behalf of
Mrs Glenys J Hunt
Chair

E-mail: nrescommittee.northwest-liverpooleast@nhs.net

Enclosures: List of names and professions of members who took part in the review

North West - Liverpool East Research Ethics Committee

Attendance at Sub-Committee of the REC meeting

Committee Members:

<i>Name</i>	<i>Profession</i>	<i>Present</i>	<i>Notes</i>
Mrs Glenys J Hunt Chair	Solicitor	Yes	
Dr Peter Walton	Lay Member	Yes	

Also in attendance:

<i>Name</i>	<i>Position (or reason for attending)</i>
Miss Ewa Grzegorska	REC Assistant

Appendix 2: Adult volunteer donor ethics

From: Ethics
Sent: 26 January 2015 17:10
To: Flanagan, Brian
Subject: RE: RETH000773: request for review of study ethics application.

Dear Dr Flanagan,

I am pleased to inform you that the Sub-Committee has approved your application for ethical approval for your study. Details and conditions of the approval can be found below.

Reference: RETH000773
Sub-Committee: Physical Interventions
Review type: Full committee review
Principle Investigator: Dr Brian Flanagan
Department: Women's and Children's Health
Title: Analysis of normal blood leukocyte function using cells from healthy volunteers.
First Reviewer: Professor Graham Kemp
Date of initial review: 11/12/2014
Date of Approval: 26/01/2015

The application was APPROVED subject to the following conditions:

Conditions

All serious adverse events must be reported to the Sub-Committee within 24 hours of their occurrence, via the Research Integrity and Governance Officer (ethics@liv.ac.uk).

This approval applies for the duration of the research. If it is proposed to extend the duration of the study as specified in the application form, the Sub-Committee should be notified. If it is proposed to make an amendment to the research, you should notify the Sub-Committee by following the Notice of Amendment procedure outlined at <http://www.liv.ac.uk/media/livacuk/researchethics/notice%20of%20amendment.doc>. If the named PI / Supervisor leaves the employment of the University during the course of this approval, the approval will lapse. Therefore please contact the Research Integrity and Governance Officer at ethics@liverpool.ac.uk in order to notify them of a change in PI / Supervisor.

Kind regards

Matthew Billington
Research Integrity and Governance Officer

Research Support Office
University of Liverpool
Waterhouse Building (2nd Floor, Block C)
3 Brownlow Street
Liverpool
L69 3GL

Email: ethics@liverpool.ac.uk
Telephone: 0151 794 8290
Website: [Research Integrity & Ethics](#)

Please note: My working hours are Monday to Friday, 8:00am - 4:00pm.

 Please ensure you are familiar with the [Research Integrity Concordat](#)

Appendix 3: Clinical forms

Appendix 3.1: JSLE clinical and BILAG forms

Appendix 3.1.1: JSLE annual assessment form

JSLE – ANNUAL ASSESSMENT (inc SLICC)																												
Study No																												
Date		/		/																								
					Retrospective	Prospective																						
					CHQ	SF36																						
*** All fields should be completed – this is the minimal annual monitoring dataset ***																												
Autoantibodies (Most recent in last 12 months – should be done at least annually)	ANA Done	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	ANA +ve	<input type="checkbox"/>	ANA titre 1:	<input type="text"/>																			
	ENA Done	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	Only tick below if positive																						
		Anti-Sm	<input type="checkbox"/>	Anti-RNP	<input type="checkbox"/>	Anti-Ro	<input type="checkbox"/>	Anti-La	<input type="checkbox"/>																			
		Other	<input type="checkbox"/>	Details <input style="width: 100%;" type="text"/>																								
Thyroid antibodies done	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	Positive	<input type="checkbox"/>	Negative	<input type="checkbox"/>																				
C1Q antibodies done	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	Positive	<input type="checkbox"/>	Negative	<input type="checkbox"/>																				
Anticardiolipin antibodies	ACA done	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	ACA-IgG	<input type="text"/>	u/ml	ACA-IgM	<input type="text"/>	u/ml																	
Lupus anticoagulant done	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	Positive	<input type="checkbox"/>	Negative	<input type="checkbox"/>																				
Glucose	<input type="text"/>	mmol/L			HbA1C	<input type="text"/>	mmol/mol																					
Liver/Muscle	AST	<input type="text"/>	iu/L	ALT	<input type="text"/>	iu/L	Albumin	<input type="text"/>	g/L	CK	<input type="text"/>	iu/L	**CMAS (if on SLICC)	<input type="text"/>														
Lipid Profile	Random	<input type="checkbox"/>	Fasting	<input type="checkbox"/>	Not Known	<input type="checkbox"/>	Cholesterol	<input type="text"/>	mmol/L	Triglycerides	<input type="text"/>	mmol/L	Apolipoproteins	<input type="checkbox"/>	ApoLPA1	<input type="text"/>	g/L	ApoLPB	<input type="text"/>	g/L	LDL/HDL	<input type="checkbox"/>	LDL	<input type="text"/>	g/L	HDL	<input type="text"/>	g/L
Thyroid Function	TSH	<input type="text"/>	mU/L	T4	<input type="text"/>	mmol/L	T3	<input type="text"/>	mmol/L																			
Ophthalmology	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	Normal	<input type="checkbox"/>	Abnormal	<input type="checkbox"/>	Date last done MM/YY:	<input type="text"/>	at Ophthalmologists	<input type="checkbox"/>	or Opticians	<input type="checkbox"/>														
DEXA every two years	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	Normal	<input type="checkbox"/>	Abnormal	<input type="checkbox"/>	Date last done MM/YY:	<input type="text"/>																		
Renal biopsy In last year	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	Nephritis class	<input type="checkbox"/>	WHO	<input type="checkbox"/>	ISN/RPS	<input type="checkbox"/>	Date last done MM/YY:	<input type="text"/>																
Puberty (using self assessment tool on pages 3&4)	Male:	Penis & scrotum score 1-5	<input type="checkbox"/>	Female:	Breasts 1-5	<input type="checkbox"/>	Pubic hair 1-5	<input type="checkbox"/>	Pre-menarche	<input type="checkbox"/>	Post-menarche	<input type="checkbox"/>																
Irregular menstruation	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	New menstrual irregularity since last visit	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>																			

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JSLE – ANNUAL ASSESSMENT (Page 2 of 2)

To be used with Glossary

A blank box will be assumed to = 0. Please tick to confirm

Yes No

Parameters	SUCC Damage Criteria	0	1	2	3
Ocular (either eye by clinic assessment)	Ocular cataract EVER				
	Retinal change OR optic atrophy				
Neuropsychiatric	Cognitive impairment OR major psychosis				
	Seizures requiring therapy for 6 months				
	Cerebral vascular accident ever (Score 2 if >1), or resection not for malignancy				
	Cranial or peripheral neuropathy (excluding optic)				
	Transverse myelitis				
Renal	Estimated or measured GFR <30%				
	Proteinuria 24h, >=3.5g OR ACR > 1000mg/mm OR > 10mg/mm				
	End stage renal disease (regardless of dialysis or transplantation)				
Pulmonary	Pulmonary hypertension (right ventricular prominence, or loud P2)				
	Pulmonary fibrosis (physical and x-ray)				
	Shrinking lung (x-ray)				
	Pleural fibrosis (x-ray)				
	Pulmonary infarction (x-ray) OR resection not for malignancy				
Cardiovascular	Angina OR coronary artery bypass				
	Myocardial infarction ever (score 2 if >1)				
	Cardiomyopathy (ventricular dysfunction)				
	Valvular disease (diastolic murmur, or a systolic murmur > 3/6)				
Peripheral Vasculer	Pericarditis x 6 months or pericardectomy				
	Claudication x 6 months				
	Minor tissue loss (pulp space)				
	Significant tissue loss ever (e.g. loss of digit or limb, resection) (Score 2 if > 1)				
Gastrointestinal	Venous thrombosis with swelling, ulceration, OR venous stasis				
	Infarction or resection of bowel (below duodenum), spleen, liver or gall bladder (Score 2 if > 1)				
	Mesenteric insufficiency				
	Chronic peritonitis				
	Stricture OR upper gastrointestinal tract surgery ever				
Musculoskeletal	Pancreatic insufficiency requiring enzyme replacement or pseudocyst				
	Atrophy or weakness (**if yes please record CMAS on page 1 of this form)				
	Deforming or erosive arthritis (including reducible deformities, excluding avascular necrosis)				
	Osteoporosis with fracture or vertebral collapse (excluding avascular necrosis)				
	Avascular necrosis (Score 2 if > 1)				
	Osteomyelitis				
	Ruptured tendons				
kin	Alopecia				
	Extensive scarring of panniculum other than scalp and pulp space				
	Skin ulceration (not due to thrombosis) > 6 months				
Other	Diabetes (regardless of treatment)				
	Malignancy (excluding dysplasia) (Score 2 if > 1)				
	Premature gonadal failure / secondary amenorrhoea				

**PUBERTY
SELF
ASSESSMENT
BOYS**

UK JSLE COHORT
STUDY NUMBER:

.....

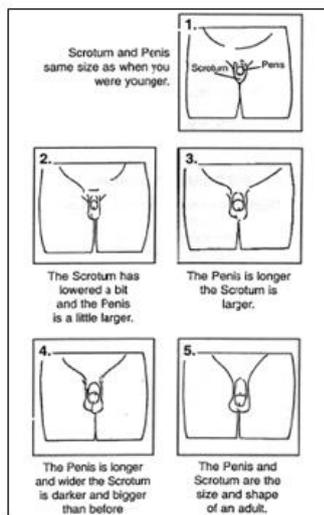
DATE:

.....

PUBERTY SELF ASSESSMENT – BOYS

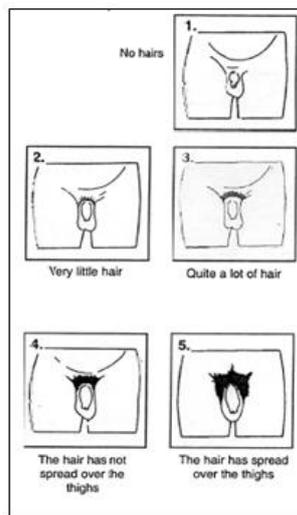
Please look at the **Penis and Scrotum** only in these pictures

- Please tick the box that looks most like you now



Please look at the **Pubic Hair** only in these pictures

- Please put a tick in the box that looks most like you now



**PUBERTY
SELF
ASSESSMENT
GIRLS**

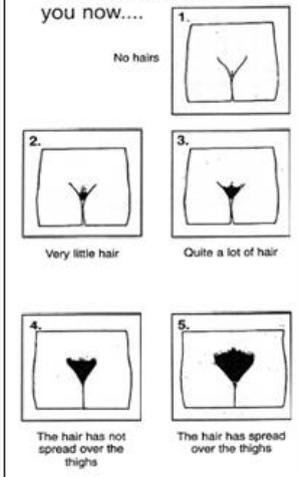
UK JSLE COHORT
STUDY NUMBER:

.....

DATE:

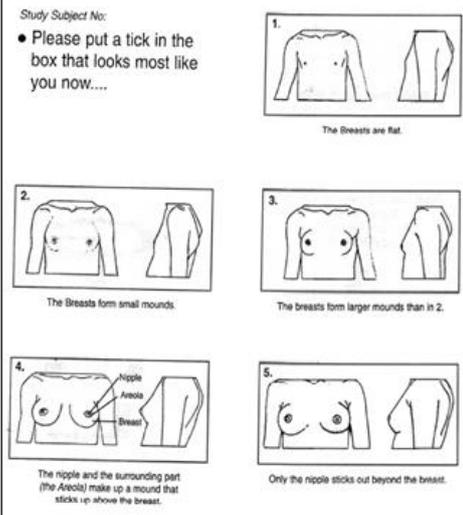
.....

- Please put a tick in the box that looks most like you now....



Study Subject No:

- Please put a tick in the box that looks most like you now....



Please answer the following questions:

1. Have you started having your period yet? Y / N
2. If yes, at what age did it start:
3. Do you have irregular periods? Y / N
4. If yes, is this a new problem since your last clinic appointment? Y/N

Appendix 3.1.2: JSLE ACR and SLICC form

SLICC CLASSIFICATION CRITERIA AT ANNUAL REVIEW										
JSLE – ACR CLASSIFICATION CRITERIA AT ANNUAL REVIEW										
Study No	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
Date	<input type="text"/>	<input type="text"/>	/	<input type="text"/>	<input type="text"/>	/	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
Retrospective						<input type="checkbox"/>	Prospective	<input type="checkbox"/>		
These are cumulative. Please add new criteria only or tick the box if no new criteria since last review <input type="checkbox"/>										
Criterion	Present	Subtype			Definition					
1 Malar Rash	<input type="checkbox"/>				Fixed erythema, flat or raised, over malar eminences, tending to spare the nasolabial folds					
2 Discoid Lupus	<input type="checkbox"/>				Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions					
3 Photosensitivity	<input type="checkbox"/>				Skin rash as a result of the unusual reaction to sunlight, by patient history or physician observation					
4 Oral/nasal ulcerations	<input type="checkbox"/>				Oral or nasopharyngeal ulceration, usually painless, observed by physician					
5 Non-erosive Arthritis	<input type="checkbox"/>				Non-erosive arthritis involving 2 or more peripheral joints, characterised by tenderness, swelling or effusion					
6 Serositis	<input type="checkbox"/>	A	<input type="checkbox"/>	Pleuritis—convincing history of pleuritic pain or rub heard, or evidence of pleural effusion Pericarditis—documented ECG or rub, or evidence of pericardial effusion						
	<input type="checkbox"/>	B	<input type="checkbox"/>							
7 Nephritis	<input type="checkbox"/>	A	<input type="checkbox"/>	Persistent proteinuria > 0.5g/day or > +++ (on protein dipstick) if quantification not performed Cellular casts – may be red cell, haemoglobin, granular, tubular or mixed						
	<input type="checkbox"/>	B	<input type="checkbox"/>							
8 Neurologic	<input type="checkbox"/>	A	<input type="checkbox"/>	Seizures in the absence of offending drugs or metabolic derangements (e.g. uraemia, ketoacidosis, electrolyte imbalance) Psychosis in the absence of offending drugs or metabolic derangements (e.g. uraemia, ketoacidosis, electrolyte imbalance)						
	<input type="checkbox"/>	B	<input type="checkbox"/>							
9 Haematological Disorder	<input type="checkbox"/>	A	<input type="checkbox"/>	Haemolytic anaemia with reticulocytes						
	<input type="checkbox"/>	B	<input type="checkbox"/>	Leucopenia < 4,000/mm ³ total on 2 or more occasions						
	<input type="checkbox"/>	C	<input type="checkbox"/>	Lymphopenia < 1,500/mm ³ on 2 or more occasions						
	<input type="checkbox"/>	D	<input type="checkbox"/>	Thrombocytopenia < 100,000/mm ³ in absence of offending drugs						
10 Immunological Disorder	<input type="checkbox"/>	A	<input type="checkbox"/>	Anti-DNA: antibody to native DNA in abnormal titre						
	<input type="checkbox"/>	B	<input type="checkbox"/>	Anti-Sm: presence of antibody to Sm nuclear antigen						
	<input type="checkbox"/>	C	<input type="checkbox"/>	+ve finding of anti-phospholipid antibodies based on:						
	<input type="checkbox"/>	C1	<input type="checkbox"/>	Abnormal level of IgG or IgM anti-cardiolipin antibody						
	<input type="checkbox"/>	C2	<input type="checkbox"/>	Positive test result for lupus anticoagulant (standard method)						
<input type="checkbox"/>	C3	<input type="checkbox"/>	False +ve serologic result for syphilis > 6 mths							
11 ANA	<input type="checkbox"/>				Abnormal titre ANA at any time point in absence of drugs known to be associated with "drug-induced lupus"					
If less than 4 criteria why evolving Lupus?		<input type="text"/>								

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Study No								Date			/		/		
These are cumulative. Please add new criteria only or tick the box if no new criteria since last review															

Criterion	Present	Subtype	Definition			
Clinical Criteria	1	Acute cutaneous lupus in the absence of dermatomyositis	<input type="checkbox"/>	A	<input type="checkbox"/>	Lupus malar rash (do not count if malar discoid)
			<input type="checkbox"/>	B	<input type="checkbox"/>	Bullous lupus
			<input type="checkbox"/>	C	<input type="checkbox"/>	Toxic epidermal necrolysis variant of SKE
			<input type="checkbox"/>	D	<input type="checkbox"/>	Meculopopular lupus rash
			<input type="checkbox"/>	E	<input type="checkbox"/>	Photosensitive lupus rash
			<input type="checkbox"/>		<input type="checkbox"/>	Subacute cutaneous lupus (Nonindurated psoriasisiform and/or annular polycyclic lesions that resolve without scarring, although occasionally with postinflammatory dyspigmentation or telangiectasia)
	2	Chronic cutaneous lupus	<input type="checkbox"/>	A	<input type="checkbox"/>	Classic discoid rash localised (above the neck)
			<input type="checkbox"/>	B	<input type="checkbox"/>	Classic discoid rash generalised (above and below the neck)
			<input type="checkbox"/>	C	<input type="checkbox"/>	Hypertrophic (verruccous lupus)
			<input type="checkbox"/>	D	<input type="checkbox"/>	Lupus panniculitis (profundus)
			<input type="checkbox"/>	E	<input type="checkbox"/>	Mucosal lupus
<input type="checkbox"/>			F	<input type="checkbox"/>	Lupus erythematosus tumidus	
<input type="checkbox"/>			G	<input type="checkbox"/>	Chilblains lupus	
<input type="checkbox"/>			H	<input type="checkbox"/>	Discoid lupus / lichen planus overlap	
3	Ulcers	<input type="checkbox"/>	A	<input type="checkbox"/>	Oral ulcers (Palate/Buccal/Tongue) in absence of other causes, such as vasculitis, Behçet's disease, infection (Herpesvirus), inflammatory bowel disease, reactive arthritis and acic/food	
		<input type="checkbox"/>	B	<input type="checkbox"/>	Nasal ulcers - in the absence of other causes, such as vasculitis, Behçet's disease, infection (Herpesvirus), inflammatory bowel disease, reactive arthritis and acic/food	
4	Nonscarring alopecia	<input type="checkbox"/>			Diffuse thinning or hair fragility with visible broken hairs in the absence of other causes such as alopecia areata, drugs, iron deficiency and androgenic alopecia	
5	Synovitis	<input type="checkbox"/>			Involving 2 or more joints, characterised by swelling or effusion OR tenderness in 2 or more joints and at least 30 minutes of morning stiffness	
6	Serositis	<input type="checkbox"/>	A	<input type="checkbox"/>	Typical pleurisy for more than 1 day OR pleural effusions OR pleural rub	
		<input type="checkbox"/>	B	<input type="checkbox"/>	Typical pericardial pain (pain with recumbency improved by sitting forward) for more than 1 day OR pericardial effusion OR pericardial rub OR pericarditis by ECG in the absence of other causes such as infection, uraemia, and Dressler's pericarditis	
7	Renal	<input type="checkbox"/>	A	<input type="checkbox"/>	Urine protein-to-creatinine ratio (or 24-hour urine protein) representing 300mg protein/24 hours	
		<input type="checkbox"/>	B	<input type="checkbox"/>	Red blood cell casts	
8	Neurological	<input type="checkbox"/>	A	<input type="checkbox"/>	Seizures	
		<input type="checkbox"/>	B	<input type="checkbox"/>	Psychosis	
		<input type="checkbox"/>	C	<input type="checkbox"/>	Mononeuritis multiplex in the absence of other known causes such as primary vasculitis	
		<input type="checkbox"/>	D	<input type="checkbox"/>	Myelitis	
		<input type="checkbox"/>	E	<input type="checkbox"/>	Peripheral or cranial neuropathy in the absence of other known causes such as primary vasculitis, infection, and diabetes mellitus	
		<input type="checkbox"/>	F	<input type="checkbox"/>	Acute confusional state in the absence of other causes, including toxic/metabolic, uraemia, drugs	
9	Haemolytic anaemia	<input type="checkbox"/>				
10	Leukopenia or lymphopenia	<input type="checkbox"/>	A	<input type="checkbox"/>	Leukopenia (<4,000/mm ³ at least once) in the absence of other known causes such as Felty's syndrome, drugs and partial hypertension	
		<input type="checkbox"/>	B	<input type="checkbox"/>	Lymphopenia (<1,000/mm ³ at least once) in the absence of other known causes such as corticosteroids, drugs and infection	
11	Thrombocytopenia	<input type="checkbox"/>			Thrombocytopenia <100,000/mm ³ at least once in the absence of other known causes such as drugs, partial hypertension, and thrombotic thrombocytopenic purpura	
Immunologic criteria	1	ANA	<input type="checkbox"/>		ANA level above laboratory reference range	
	2	Anti-dsDNA	<input type="checkbox"/>		Anti-dsDNA antibody level above laboratory reference range (or >2-fold the reference range if tested by ELISA)	
	3	Anti-Sm	<input type="checkbox"/>		Presence of antibody to Sm nuclear antigen	
	4	Antiphospholipid antibody positivity	<input type="checkbox"/>	A	<input type="checkbox"/>	Positive test result for lupus anticoagulant
			<input type="checkbox"/>	B	<input type="checkbox"/>	False-positive test result for rapid plasma reagin
			<input type="checkbox"/>	C	<input type="checkbox"/>	Medium- or high-titer anticardiolipin antibody level (IgA, IgG, or IgM)
<input type="checkbox"/>			D	<input type="checkbox"/>	Positive test result for anti-β ₂ -glycoprotein I (IgA, IgG, or IgM)	
5	Low complement	<input type="checkbox"/>	A	<input type="checkbox"/>	Low C3	
		<input type="checkbox"/>	B	<input type="checkbox"/>	Low C4	
		<input type="checkbox"/>	C	<input type="checkbox"/>	Low CH50	
6	Direct Coombs' test	<input type="checkbox"/>			In the absence of haemolytic anaemia	

Appendix 3.1.3 JSLE BILAG form

JSLE – BILAG Form

Study No

Date / /

Routine	Flare
<input type="checkbox"/>	<input type="checkbox"/>
Retrospective	Prospective
<input type="checkbox"/>	<input type="checkbox"/>

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

Height cm

Weight kg

Systolic BP mmHg

Diastolic BP mmHg

Urinalysis (score 0, trace, 1+, 2+, 3+).
 If abnormal urinalysis send urine for Microscopy, culture, sensitivities (MCS) Not Done

Proteinuria Haematuria Leucocytes Nitrites Menstruating Y / N

NB. The following bloods should be sent as part of BILAG: FBC, ESR, CRP, C3, C4, dsDNA, total Igs, Creatinine, urinary protein/Cr or Alb/Cr ratio. Yes No
 Please tick to confirm done

To be used with Glossary: * = Definition in Glossary
 Only features attributable to JSLE to be recorded and refer only to last 4 week compared with previous 4 weeks

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions). A blank box will be assumed to = 0 or No. Yes No
 Please tick to confirm

General	
* 1	Pyrexia (documented >37.5°C)
* 2	Weight Loss – unintentional; >5%
* 3	Lymphadenopathy/splenomegaly
4	Fatigue/malaise/lethargy
5	Anorexia
Mucocutaneous	
* 6	Skin eruption – severe active (not discoid/bullous/panniculitis)
* 7	Skin eruption – mild
* 8	Active discoid lesions: generalised/extensive
* 9	Active discoid lesions: localised include lupus profundus
* 10	Alopecia (severe, active)
* 11	Alopecia (mild)
* 12	Panniculitis/bullous lupus (severe)
* 13	Panniculitis/bullous lupus (mild)
* 14a	Angio-oedema (severe)
* 14b	Angio-oedema (mild)
* 15	Mucosal ulceration (severe)
* 16	Mucosal ulcers (mild)
* 17	Malar erythema
18	Subcutaneous nodules

19	Peri-otic skin lesions		
* 20	Peri-ungual erythema / chilblains		
21	Swollen fingers	Y	N
22	Sclerodactyly	Y	N
23	Calcinosis	Y	N
24	Telangiectasia	Y	N
25	Splinter haemorrhages	Y	N
Neurological			
26	Impaired level of consciousness		
* 27	Cognitive dysfunction		
* 28	Acute psychosis or delirium or confusional state		
* 29	Psychosis		
* 30	Seizure disorder		
* 31	Status epilepticus		
* 32	Cerebral vascular disease (not due to vasculitis)		
* 33	Cerebral vasculitis		
* 34	Aseptic meningitis		
* 35	Mononeuropathy (single/multiplex)		
36	Ascending or transverse myelitis		
* 37	Demyelinating syndrome		
* 38	Myelopathy		

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Page 1 of 5

JSLE – BILAG Form

Study No					
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Date									
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For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

Neurological continued					
* 39	Acute inflammatory demyelinating polyradiculoneuropathy				
40	Peripheral neuropathy				
* 41	Cranial neuropathy				
* 42	Plexopathy				
* 43	Polyneuropathy				
* 44	Autonomic disorder				
* 45	Disc swelling				
* 46	Chorea				
* 47	Cerebellar ataxia (isolated)				
* 48	Movement disorder				
* 49	Lupus headache (severe, unremitting)				
* 50	Episodic migrainous headaches				
* 51	Tension headache				
* 52	Cluster headache				
* 53	Headache from IC hypertension				
* 54	Organic depressive illness				
* 55	Mood disorder (depression/mania)				
* 56	Anxiety disorder				
* 57	Organic brain syndrome				
Musculoskeletal					
* 58	Definite myositis (severe)				
* 59	Myositis with incomplete criteria				
* 60	Myositis (mild)				
* 61	Myalgia				
* 62	Severe polyarthritis – with loss of function				
* 63	Moderate arthritis				
* 64	Arthralgia				
* 65	Tendonitis/tenosynovitis				
66	Tendon contractures and fixed deformity	Y			N

67	Aseptic necrosis	Y			N
Cardiovascular & Respiratory					
* 68	Pleuropericardial pain				
* 69	Dyspnoea				
* 70	Cardiac failure				
* 71	Friction rub				
* 72	Effusion (pericardial or pleural)				
* 73	Mild or intermittent chest pain				
74	Progressive CXR changes – lung fields	Y			N
75	Progressive CXR changes – heart size	Y			N
76	ECG evidence of pericarditis/myocarditis/endocarditis	Y			N
* 77	Cardiac arrhythmia including tachycardia (>100 no fever)	Y			N
* 78	Pulmonary function fall by >20%	Y			N
79	Cytohistological evidence of inflammatory lung disease	Y			N
* 80	Myocarditis - mild				
* 81	New valvular dysfunction				
* 82	Cardiac tamponade				
* 83	Pleural effusion with dyspnoea				
* 84	Pulmonary haemorrhage/vasculitis				
* 85	Interstitial alveolitis/pneumonitis				
* 86	Shrinking lung syndrome				
* 87	Aortitis				
* 88	Coronary vasculitis				
Vasculitis					
* 89	Major cutaneous vasculitis including ulcers				
* 90	Major abdominal crisis due to vasculitis				
91	Recurrent thromboembolism (excluding strokes)				
92	Raynaud's				
93	Livido reticularis				

JSLE – BILAG Form

Study No		Date			
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For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

Vasculitis continued				
94	Superficial phlebitis			
* 95	Minor cutaneous vasculitis (nailfold, digital, purpura, urticaria)			
96	Thromboembolism (excl stroke) 1 st episode	Y		N
Renal				
* 99	Severe hypertension	Y		N
103	Newly documented proteinuria (level 4)	Y		N
* 104	Nephrotic syndrome	Y		N
* 108	Histological evidence of active nephritis within 3 months	Y		N
Gastrointestinal				
* 109	Lupus peritonitis			
* 110	Abdominal serositis or ascites			
* 111	Lupus enteritis/colitis			
* 112	Malabsorption			
* 113	Protein losing enteropathy			
* 114	Intestinal pseudo-obstruction			
* 115	Lupus hepatitis			

* 116	Acute lupus cholecystitis			
* 117	Acute lupus pancreatitis			
Ophthalmic				
* 118	Orbital inflammation with myositis and/or proptosis			
* 119	Keratitis – severe			
* 120	Keratitis – mild			
121	Anterior uveitis			
* 122	Posterior uveitis/retinal vasculitis – severe			
* 123	Posterior uveitis/retinal vasculitis – mild			
124	Episcleritis			
* 125	Scleritis – severe			
* 126	Scleritis – mild			
* 127	Retinal / choroidal vaso-occlusive disease			
* 128	Isolated cotton-wool spots (cytoid bodies)			
* 129	Optic neuritis			
* 130	Anterior ischaemic optic neuropathy			

Function

Patient CHAQ *

Global assessment on CHAQ (0: Very well – 100: Very ill)

Physicians clinical global score Very well 0 100 Very ill

Important assessment of current clinical status

In relation to the patients LUPUS ACTIVITY at this time point, please tick the box that best describes your opinion as to their current flare status:

1. Clinical improvement – no clinical flare
2. No change in Lupus activity
3. Minor flare (may not require specific therapy)
4. Moderate flare
5. Major flare

*A flare of JSLE has been defined as "a measurable worsening of SLE disease activity in at least one organ system, involving new or worse signs of disease that may be accompanied by new or worse JSLE symptoms; depending on the severity of the flare, more intensive therapy may be required"*¹

1. Brunner H, Klein-Gitelman MS, Higgins GC, et al. Arthritis Care Res. 2010;52(6):811-20.

JSLE – BILAG Form

Study No		Date	
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For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = now; 0 = not present (except for Y/N and numeric questions).

Treatment	Current dose	Revised dose
Hydroxychloroquine (mg/day)	[][][]	[][][]
Azathioprine (mg/day)	[][][]	[][][]
Mycophenolate (mg/day)	[][][][]	[][][][]
Cyclosporin (mg/day)	[][][]	[][][]
Prednisolone (mg/day)	[][] • []	[][] • []
Methotrexate (mg/week)	[][] • []	[][] • []
	Oral <input type="checkbox"/>	Oral <input type="checkbox"/>
	Subcut <input type="checkbox"/>	Subcut <input type="checkbox"/>
	Current dose	Total number of pulses since last visit
IVIg (g/pulse)	[][][] • []	[][]

Rituximab: Total dose per cycle (mg) [][][][][] Number of infusions per cycle [][] Number of cycles since last visit [][]	Cyclophosphamide: IV <input type="checkbox"/> Oral <input type="checkbox"/> Total dose since last visit (mg) [][][][][] Number of infusions since last visit [][] Cumulative dose [][][][][]
IVI methyl-prednisolone in last 3 months	<5 pulses [] >=5 pulses []

Other Drugs	Aspirin <input type="checkbox"/>	Bisphosphonate <input type="checkbox"/>	Oral contraceptive pill <input type="checkbox"/>
	Angiotensin receptor blocker <input type="checkbox"/>	Ca ⁺⁺ blockers <input type="checkbox"/>	Statins <input type="checkbox"/>
	Other biological DMARDS <input type="checkbox"/>	ACEi <input type="checkbox"/>	Diuretic <input type="checkbox"/>
			Anticoagulant <input type="checkbox"/>

Clinicians Intention re Medication
 Please tick which of the following best describes your intention to change treatment, and explain why:

1) Decrease in treatment <input type="checkbox"/> <ul style="list-style-type: none"> - Disease improvement <input type="checkbox"/> - Side effects of treatment <input type="checkbox"/> - Compliance problems <input type="checkbox"/> - Weaning regimen <input type="checkbox"/> 	3) Change in DMARD <input type="checkbox"/> <ul style="list-style-type: none"> - Concerns over efficacy <input type="checkbox"/> - Planned maintenance treatment <input type="checkbox"/> - Side effects of treatment <input type="checkbox"/> - Compliance problems <input type="checkbox"/>
2) Increase in treatment <input type="checkbox"/> <ul style="list-style-type: none"> - Disease worsening <input type="checkbox"/> - Standard dose increment <input type="checkbox"/> - Dose increment due to weight <input type="checkbox"/> 	4) No change in treatment <input type="checkbox"/> <ul style="list-style-type: none"> - Active disease (induction phase) <input type="checkbox"/> - Stable, not yet decrease <input type="checkbox"/> - Patients choice <input type="checkbox"/> - Not on Medication <input type="checkbox"/>

JSLE – BILAG Form

Study No		Date	
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For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = now; 0 = not present (except for Y/N and numeric questions).

Results Page

Microscopy & culture results

MCS results	1. Proven UTI (>1x10 ⁵ growth (cfu) of a single organism)	Y / N	2. Mixed growth / contamination	Y / N
	3. Microscopy results:			
	a) White cell count	(per hpf)	b) Red cell count	(per hpf)
	c) Red cell casts	Y / N	d) White cell casts	Y / N

Renal function

* 102a	Urinary Alb/Cr ratio (mg/mmol Cr)	[] [] [] []	•	[]	105	Creatinine (plasma/serum)	[] [] [] []
* 102b	Urinary Protein/Cr Ratio (mg/mmol Cr)	[] [] [] []	•	[]	106a	GFR:EDTA clearance (exact) (mls/min. 1.73m ²)	[] [] [] [] • [] []
102c	24hr/Urinary Protein(g)	[] [] [] []	•	[]	106b	GFR: ht/creat ratio (estimate)	[] [] [] [] • [] []
	Not done	[]			107	Active urinary sediment	Y [] [] N

Haematology

* 131	Haemoglobin g/dl	[] []	•	[] []	* 135	Platelets x 10 ⁹ /l	[] [] [] []
* 132	Total white cell count x 10 ⁹ /l	[] [] [] []	•	[] [] [] []	* 136	Evidence of active haemolysis	Y [] [] N
* 133	Neutrophils x 10 ⁹ /l	[] [] [] []	•	[] [] [] []	137	Coomb's test positive	Y [] [] N
134	Lymphocytes x 10 ⁹ /l	[] [] [] []	•	[] [] [] []	* 138	TTP	Y [] [] N

Other measures of disease activity

ESR (mm/hr)	[] [] [] []	dsDNA	[] [] [] [] • []
CRP	[] [] [] []	IgG	[] [] • [] []
C3 (g/l)	[] [] • [] []	IgA	[] [] • [] []
C4 (g/l)	[] [] • [] []	IgM	[] [] • [] []
Viscosity (mPa.s) if no ESR	[] • [] []	Ferritin (µg/L)	[] [] [] [] [] []

Appendix 3.2: Demographic forms

Appendix 3.2.1: JSLE patient demographic form

JSLE – DEMOGRAPHICS																	
Study No	<input type="text"/>	Date	<input type="text"/>	<input type="text"/>	/	<input type="text"/>	/	<input type="text"/>	Retrospective	<input type="checkbox"/>	Prospective	<input type="checkbox"/>					
NHS Number	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	Gender	Male	<input type="checkbox"/>	Female	<input type="checkbox"/>							
Post Code	<input type="text"/>	DOB	<input type="text"/>	<input type="text"/>	/	<input type="text"/>	/	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>				
Referral	Paediatrician	<input type="checkbox"/>	GP	<input type="checkbox"/>	Adult Rheum	<input type="checkbox"/>	A&E	<input type="checkbox"/>	Sub-specialist	<input type="checkbox"/>	Sub-specialist Details						
												<input type="text"/>					
Age																	
Onset of Symptoms	Years	<input type="text"/>	<input type="text"/>	Months	<input type="text"/>	<input type="text"/>	OR	Date (if exact date not known use 01/mm/yyyy)									
Presentation	<input type="text"/>	OR	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>						
Diagnosis	<input type="text"/>	OR	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>						
Ethnicity																	
White & Black Caribbean		<input type="checkbox"/>	White & Black African		<input type="checkbox"/>	British		<input type="checkbox"/>	Irish		<input type="checkbox"/>	Any other White		<input type="checkbox"/>	Any other Mixed		<input type="checkbox"/>
Indian		<input type="checkbox"/>	Pakistani		<input type="checkbox"/>	Bangladeshi		<input type="checkbox"/>	African		<input type="checkbox"/>	Any other Asian		<input type="checkbox"/>	Any other Black		<input type="checkbox"/>
Chinese		<input type="checkbox"/>	Other		<input type="checkbox"/>	<input type="text"/>											
PMHx/EHx																	
	Self	Mother	Father	Brother	Sister	Aunt/Uncle	Grandparent										
SLE	<input type="checkbox"/>																
Thyroid	<input type="checkbox"/>																
RA	<input type="checkbox"/>																
CTD	<input type="checkbox"/>																
Type 1 DM	<input type="checkbox"/>																
Other	<input type="checkbox"/>																
None	<input type="checkbox"/>																
Details												<input type="text"/>					
Parental Consanguinity												Yes	<input type="checkbox"/>	No	<input type="checkbox"/>	Unknown	<input type="checkbox"/>
Possible Triggers																	
Infections		<input type="checkbox"/>	(Specify)	<input type="text"/>													
Medication		<input type="checkbox"/>	(Specify)	<input type="text"/>													
Sun		<input type="checkbox"/>															
Other		<input type="checkbox"/>	(Specify)	<input type="text"/>													

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Appendix 3.2.2: Paediatric control patient demographic form

CONTROL DEMOGRAPHICS

Study Number: _____ Gender: Male Female
 DOB: __ / __ / ____ Post Code: _____ Date Consented: __ / __ / ____

Ethnicity (please tick)

White British	White & Black Caribbean	Indian	Caribbean	Chinese
White Irish	White & Black African	Pakistani	African	Other: _____
Any other white background	White & Asian	Bangladeshi	Any other Black background	
	Any other mixed background	Any other Asian background		

Type of Control

JIA		Renal		Non Inflammatory	
-----	--	-------	--	------------------	--

Diagnosis (see list on next page)

(JIA only)			
	Age (Years/Months)	OR	Date
Onset of Symptoms	_____	OR	__/__/__
Presentation	_____	OR	__/__/__
Diagnosis	_____	OR	__/__/__

Has there been a family history of SLE, Thyroid problems, RA, CTD, IDDM, renal disease or any other related diseases? Yes No

	Renal Disease	Autoimmune Disease	Other
Mother			
Father			
Brother			
Sister			
Aunt / Uncle			
Grandparents			

Diagnosis

JIA:	Systemic Oligoarthritis Oligoarthritis (Extended) Oligoarthritis (Persistent) Polyarthritis Polyarthritis (Rheumatoid Factor Positive) Polyarthritis (Rheumatoid Factor Negative) Psoriatic Enthesitis related Other – meeting more than one criteria Other – not meeting other JIA criteria Undetermined
Renal:	Diabetic nethro nephropathy Nephrotic syndrome HSP nephritis Renal dysplasia Renal scarring Other
Non Inflammatory	Minor operation Dental surgery Infusion Other

Appendix 3.3: Consent forms and information sheets

Appendix 3.3.1: JSLE patient/parent information sheet



**UK Children's
Lupus Study**
Information sheet
for Parents
(JSLE patients- Liverpool only)

Why is this being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

We would like to learn more about how Lupus affects children and their treatment. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare children with Lupus to children without. Specifically we want to compare blood cells to see why Lupus cells are reacting against themselves. We are also looking at urine to help understand the Kidney problems seen.

This project is part of a UK wide study of children with Lupus.

Why are we asking your child to take part?

Because they have Lupus.

UK JSLE Cohort Study B Repository
Info Sheets Version 4 - 1st January 2016

Does your child have to take part?

No, it's up to you and your child. Either of you can change your mind at any point without giving a reason. This will not affect their care.

What will your child be asked to do?

- Your child's doctor will collect information about your child's age, gender and how they are when visiting hospital.
- When your child is having their usual blood tests we will collect a little extra blood (2-3 teaspoons) to:
 - Measure antibodies and white cells (these fight infection)
 - Study the genetics of Lupus
 - Study the Kidney involvement
- If your child has only just been diagnosed we will collect an extra sample of blood before starting treatment and at one week, one month and three months of starting treatment.
- We will also ask if your child can provide a urine sample.
- When Lupus affects the kidney, it may be necessary for your child to have a 'kidney biopsy'. This is where a

bit of kidney tissue is removed and looked at under the microscope. For this study, we would ask to be able to use any extra sample which is left over after all the standard tests have been done. This sample would be labelled with your child's study code and stored at the University of Liverpool, Alder Hey Children's Hospital. DNA (the chemical which contains your genes) will also be extracted from this sample.

- If your child has previously had a kidney biopsy we will ask your local hospital if they have enough to provide us with a piece of this.
- We will tell your child's GP they are helping us with the study.
- We will tell the Health and Social Care Information Centre that you are in the study. They collect information on all people in the UK e.g. if someone dies or develops cancer. If such an event happened, they would let us know about it.
- We will ask for an email address
- We will be in touch about future studies in Lupus to see if your child is interested in taking part.

Will it do some good if you say yes?

By doing experiments on the samples provided, your child will help us to improve understanding of Lupus and to create better treatments.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small and your child will not feel any different. Helping us with the study will not affect your child's hospital care.

What will happen to the results of the research study?

Everything we discover will be published in medical journals. Your child will not be identified in any way. Your child's test results (e.g. genetic tests) will not be fed back to you.

Who is organising the research?

It is being organised by a UK wide group of doctors and nurses called the "UK Lupus Study Group". It is run from Alder Hey Children's NHS Foundation Trust.

UK JSLE Cohort Study B Repository
Info Sheets Version 4 - 1st January 2016

The research is funded by the Alder Hey Children's NHS Foundation Trust and its Research and Development Department, the charity Lupus UK and The Alder Hey Kidney Fund.

No one receives any payment for being involved in this study.

Has the study been checked?

The Liverpool Paediatric Research Ethics Committee has approved this study.

What will happen to the information collected?

- All information and samples collected will be strictly confidential and anonymised.
- All your child's forms will be kept in the hospitals research office in a locked filing cabinet. We will keep your child's name and hospital details on a list in the research office so we know they are in the study. The office will be locked when non-attended.
- All information kept on study computers (in offices of the UK JSLE Study Group at the University of Liverpool) will only record data using a study number and will be strictly confidential. Details identifying your

child will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.

If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your child's doctor or contact:

Professor Michael Beresford
Chief Investigator UK JSLE Study Group
Alder Hey Children's NHS Foundation Trust
0151 252 5153
m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

Appendix 3.3.2: JSLE patient/parent consent form

Parental Consent Form

(Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 - 1 st January 2016) for the above study and have had the chance to ask questions	
2.	I understand my child's taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected	
3.	I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my child's blood may be used to investigate the immune system	
5.	I agree that a small amount of my child's blood may be collected and then gifted to the "UK JSLE Study Group." It will be stored for future genetic studies. I understand that no result on my child's genes will be fed back to them or anyone else.	
6.	I agree that a small amount of my blood and urine samples may be used to look into ways of detecting kidney damage in lupus.	
7.	I agree that if my child were to require a kidney biopsy in the future, then a small amount of left over kidney tissue could be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital	
8.	To be completed if your child has had a kidney biopsy in the past: I agree that a small amount of left over kidney tissue from my child's previous kidney biopsy can be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital.	
9.	I agree that DNA can be extracted and stored from any kidney biopsy sample my child provides. I understand that no result on my genes will be fed back to me or anyone else.	
10.	I agree to allow researchers to make contact with me and my child about other studies or a follow-up of this study through my child's doctors and my child's NHS number	
11.	I give permission for my child's GP to be informed that information about my child is to be held on the study database	

12.	I agree to my child being flagged with the Health and Social Care Information Centre. I understand that information held and managed at the Health and Social Care Information Centre and other central UK NHS bodies may be used in order to help contact me or provide information about my health status.	
13.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
14.	I agree to provide an email address. I understand that this will be stored securely. I understand it will only be used to provide information about the study such as the newsletter and to provide a way for the study group to contact us for follow up in the future.	
15.	I agree for my child to take part in the above study	

Name of patient

Name of person with parental
responsibility for patient

Date

Signature

Name of person taking consent
(if different from researcher)

Date

Signature

Researcher

Date

Signature

1 copy for patient and person with parental responsibility, 1 for researcher, 1 to be kept with hospital notes

Appendix 3.3.3: JSLE patient information sheets

Appendix 3.3.3.1: JSLE patient information sheet for under 6



UK Children's Lupus Study

Information sheet which can be read to patients aged less than 6 years

(JSLE patients - All centres, including Liverpool & GOSH)

Note to reader: Please read the whole sheet to yourself before reading it to your child

Your doctor has told you that you have Lupus.

Doctors want to find out why some children get Lupus, and others don't.

By finding out more about why children get Lupus we hope to be able to make better medicines.

We are doing this all over the country with children who have Lupus.

What will happen if I say yes?

- We would collect information about how you are
- We will take a small sample of your blood (2-3 teaspoons) when it is already being taken. There are NO extra needles!
- We will also ask for a urine sample

Do I have to help?

No, not if your mum or dad or the grown up looking after you decide that they don't want you to. You will be looked after just the same.

Will it do some good if I say yes?

Yes you will be helping us make better treatments for Lupus

What do I have to do now?

We are asking you to say "Yes" or "No" to the question:

"Can we collect information about how you are and a small blood and urine sample?"

What if I have questions?

If you have any questions you or your mum and dad or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!



UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Appendix 3.3.3.2: JSLE patient information sheet for 6-12



UK Children's Lupus Study

Information sheet
for patients aged 6-
12 years

(JSLE patients - Liverpool & GOSH only)

Why is this being done?

This research is looking at what causes Lupus. We don't fully understand this, especially in children.

We hope that by understanding more about the causes of Lupus we can make better treatments.

To do this we need to compare children with Lupus, like you, to children without Lupus.

Why have I been chosen?

You are being asked to take part in this study because you have Lupus.

What will happen if I say yes?

We will ask you to write your name on a form. This is to say that you understand what the study is about and what will happen.

We would like to collect information about how you are.

We will take a small sample of your blood (2-3 teaspoons) when it is already being taken. There are NO extra needles!

We will also ask for a urine sample.

If Lupus affects your kidneys your doctor may have done / or be planning to do some tests on a little bit of your kidney tissue. We would ask your doctor for a bit of 'left over' kidney tissue as part of this study.

Do I have to help?

No, not if your mum or dad or the grown up looking after you decide that they do not want you to. You will be looked after just the same.

Will it do some good if I say yes?

By looking at the samples given, you will be helping us to treat children with Lupus, like you, better in the future.

Will anything bad happen to me if I take part?

No, nothing bad will happen to you. All information that is collected about you will be kept private between you / your parents and the study organisers.

What do I have to do now?

If you want to help, you and your mum, or dad or the grown up looking after you will have to say it's ok.

What if I have questions?

If you have any questions you or your mum or dad or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this
leaflet!



Appendix 3.3.3.3: JSLE patient information sheet for 13-15



**UK Children's
Lupus Study**
Information sheet
for patients aged
13-15 years |
(JSLE patients - Liverpool only)

Why is this study being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

We would like to learn more about how Lupus affects children and their treatment. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare the blood cells and urine of children with and without Lupus. This will give us information on why Lupus cells are reacting against themselves and the kidney problems seen in Lupus.

Why am I being asked to take part?

Because you have Lupus.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Do I have to take part?

No, it's up to you. You can change your mind at any point without giving a reason. This will not affect your hospital care.

What will happen if I say yes?

- Your doctor will collect information about your age, gender and how you are.
- When you are having your usual blood tests we will collect a little extra blood (2-3 teaspoons). We will use this to:
 - Measure your autoantibodies and white cells (these usually fight infection)
 - Study the genetics of Lupus
 - Study the kidney involvement
- If you have only just been diagnosed we will collect an extra sample of blood before you start treatment and after one week, one month and three months of starting treatment.
- We will also ask if you can give us a urine sample.

- When Lupus affects the kidney, it may be necessary to have a 'kidney biopsy'. This is where a bit of kidney tissue is removed and looked at under the microscope. We would like to have any extra sample, which is left over after all the standard tests have been done. This sample would be stored at the University of Liverpool. DNA (contained in your genes) in the kidney sample will also be tested.
- If you have had a kidney biopsy in the past we will ask your local hospital if they have enough to provide us with a small piece of this.
- We will tell your GP you are in the study.
- We will tell the Health and Social Care Information Centre that you are in the study. They collect health information on all people in the UK e.g. if someone dies or develops cancer.
- We will ask for an email address
- We will be in touch about any future studies in Lupus to see if you are interested in taking part.

Will it do some good if I say yes?

By doing experiments on your samples you will help us to improve understanding of Lupus.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small. Helping us with the study will not affect your hospital care.

What will happen to the results of the research study?

Everything we discover will be presented in scientific meetings and published in medical journals. This may take several years.

Individual test results (e.g. genetic tests) will not be fed back to you.

Who is organising the research?

It is being organised by a UK wide group of doctors and nurses called the "UK Lupus Study Group".

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's

Research and Development Department,
Lupus UK and The Alder Hey Kidney Fund.

No one, including your doctor receives any payment for being involved.

Has the study been checked?

The Liverpool Research Ethics Committee has approved this study.

What will happen to the information collected about me?

All information and samples collected from you will be strictly confidential and anonymised.

All your study forms will be kept in your hospital's research office, stored in a locked filing cabinet. Your name and hospital details will be kept on a list in the research office so we know you are in the study. This office is locked when not-attended, for confidentiality.

All information kept on study computers (at the University of Liverpool) will only record data using your study number and be strictly confidential. Details identifying who you are will not be kept on the study

computer. All electronic transfer of data will use codes.

What if I have questions?

If you have any questions you, your mum / dad or the grown up looking after you can speak to the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford
Chief Investigator UK JSLE Study Group
Alder Hey Children's NHS Foundation Trust
0151 252 5153
m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!



UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Appendix 3.3.3.4: JSLE patient information sheet for 16 and over



**UK Children's
Lupus Study**
Information sheet
for patients aged 16
years and older
(JSLE patients - Liverpool only)

Why is this study being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

We would like to learn more about how Lupus affects children and their treatment. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare the blood cells of children with and without Lupus, to see why Lupus cells are reacting against themselves. We are also looking at urine to better understand the kidney problems seen in Lupus.

Why am I being asked to take part?

Because you have Lupus.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Do I have to take part?

No, it's up to you. You can change your mind at any point without giving a reason. This will not affect your hospital care.

What will happen if I say yes?

- Your doctor will collect information about your age, gender and how you are when you visit the hospital.
- When you are having your usual blood tests we will collect a little extra blood (2-3 teaspoons). We will use this to:
 - Measure your autoantibodies and white cells (these usually fight infection)
 - Study the genetics of Lupus
 - Study the kidney involvement
- If you have only just been diagnosed we will collect an extra sample of blood before you start treatment and after one week, one month and three months of starting treatment.
- We will also ask if you can give us a urine sample.

- When Lupus affects the kidney, it may be necessary to have a 'kidney biopsy'. This is where a bit of kidney tissue is removed and looked at under the microscope. We would like to have any extra sample which is left over after all the standard tests have been done. This sample would be stored at the University of Liverpool, Alder Hey Children's Hospital. DNA (contained in your genes) in the kidney sample will also be tested.
- If you have had a kidney biopsy in the past we will ask your local hospital if they have enough to provide us with a small piece of this.
- We will tell your GP you are in the study.
- We will tell the Health and Social Care Information Centre that you are in the study. They collect information on all people in the UK e.g. if someone dies or develops cancer. If such an event happened, they would let us know about it.
- We will ask for an email address
- We will be in touch about any future studies in Lupus to see if you are interested in taking part.

Will it do some good if I say yes?

By doing experiments on your samples you will help us to improve understanding of Lupus and create better treatments.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small. Helping us with the study will not affect your hospital care.

What will happen to the results of the research study?

Everything we discover will be presented in scientific meetings and published in medical journals. This may take several years. Individual test results (e.g. genetic tests) will not be fed back to you.

Who is organising the research?

It is being organised by a UK wide group of doctors and nurses called the "UK Lupus Study Group". It is run from Alder Hey Children's Hospital.

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's

Research and Development Department, Lupus UK and The Alder Hey Kidney Fund.

No one, including your doctor receives any payment for being involved.

Has the study been checked?

The Liverpool Research Ethics Committee has approved this study.

What will happen to the information collected about me?

All information and samples collected from you will be strictly confidential and anonymised.

All your study forms will be kept in your hospital's research office, stored in a locked filing cabinet. Your name and hospital details will be kept on a list in the research office so we know you are in the study. This office is locked when not-attended, for confidentiality.

All information kept on study computers (at the University of Liverpool) will only record data using your study number and be strictly confidential. Details identifying who you are will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.

If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford
Chief Investigator UK JSLE Study Group
Alder Hey Children's NHS Foundation Trust
0151 252 5153
m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Appendix 3.3.4: JSLE patient assent form

Assent Form - Patients

(Liverpool and GOSH only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have been told about the study and given the information sheet (Version 4 - 1 st January 2016) for the above study and have had the chance to ask questions	
2.	I understand taking part is voluntary and I can stop taking part at any time, without giving any reason, without this making any difference to my medical care or legal rights	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust, where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records	
4.	I agree that a small amount of my blood and in some cases urine may be used for research.	
5.	I agree that if I need a kidney biopsy in the future, then a small amount of left over kidney tissue could be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital, to look at how the kidney is affected by Lupus.	
6.	To be completed by patients who have had a kidney biopsy in the past: I agree that a small amount of left over kidney tissue from my previous kidney biopsy can be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital.	
7.	I agree that DNA can be extracted and stored from any kidney biopsy samples that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
8.	I agree that I will take part in the above study	
9.	I agree to allow researchers to make contact with me about other studies or a follow-up of this study through my doctors and NHS number	
10.	I give permission for my GP to be informed that information about me is to be held on the study database	
11.	I agree to being flagged with the Health and Social Care Information Centre. I understand that information held and managed at the Health and Social Care Information Centre and other central UK NHS bodies may be used in order to help contact me or provide information about my health status.	
12.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	

Patient's Consent Form

(Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 -1 st January 2016) for the above study and have had the chance to ask questions	
2.	I understand taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records	
4.	I agree that a small amount of my blood may be used to investigate the immune system	
5.	I agree that a small amount of my blood may be collected and gifted to the "UK JSLE Study Group." It will be stored for future genetic studies. I understand that no result on my genes will be fed back to me or anyone else.	
6.	I agree that a small amount of my blood and urine samples may be used to look into ways of detecting kidney damage in lupus.	
7.	I agree that if I was to need a kidney biopsy in the future, then a small amount of left over kidney tissue could be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital, to look at how the kidney is affected by Lupus.	
8.	To be completed by patients who have had a kidney biopsy in the past: I agree that a small amount of left over kidney tissue from my previous kidney biopsy can be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital.	
9.	I agree that DNA can be extracted and stored from any kidney biopsy samples that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
10.	I agree to allow researchers to make contact with me about other studies or a follow-up of this study through my doctors and my NHS number	
11.	I give permission for my GP to be informed that information about me is to be held on the study database	

Appendix 3.3.6: Paediatric control patient/parent information sheet



UK Children's Lupus Study
Information sheet
for Parents
(Paediatric controls - Liverpool only)

Why is this being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare children with Lupus to children without Lupus, like your child. Specifically we need to compare blood cells to see why Lupus cells are reacting against themselves and urine to detect inflammation of the kidney, a common complication of Lupus.

This project is part of a UK wide study of children with Lupus.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Why are we asking your child to take part?

Because they do not have Lupus.

Does your child have to take part?

No, it's up to you and your child. You and your child can change your mind at any point without giving a reason. This will not affect the care they receive.

What will your child be asked to do?

- We will take a sample of your child's blood (2-3 teaspoons). If your child needs a blood test for another reason we will take it at the same time.
- We will also ask if your child can provide a urine sample. We use this to look at how the kidneys work in Lupus.
- We will collect information about your child's age, gender and your child's general health. Your child's participation in the study will be kept confidential.

- We will keep your child's name and hospital details on a list in the hospital's locked research office so we know you are in the study.
- We will tell your GP you are helping us with the study

Will it do some good if we say yes?

By doing experiments on the samples given by your child, this will help us create better treatments for children with Lupus.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small. Helping us with the study will not affect their care.

What will happen to the results of the research study?

Everything we discover will be presented at scientific meetings and published in medical journals. Your child will not be identified in any way.

Who is organising the research?

It is being organised by a group of doctors and nurses from around the country called the "UK Lupus Study Group". It is run from Alder Hey Children's NHS Foundation Trust.

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's Research and Development Department at Alder Hey, the charity Lupus UK and The Alder Hey Kidney Fund.

No one, including your doctor receives any payment for being involved in this study.

Has the study been checked?

The Liverpool Paediatric Research Ethics Committee has approved this study.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

What will happen to the information collected?

All information and samples collected will be strictly confidential and anonymised.

Forms will be kept in the hospital's research office. All forms will be stored in locked filing cabinets in rooms that are locked when non-attended.

All information kept on study computers (in offices of the UK JSLE Study Group at the Institute Child Health, University of Liverpool) will only record data using a study number and will be strictly confidential. Details identifying your child will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.

If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford
Chief Investigator UK JSLE Study Group
Alder Hey Children's NHS Foundation Trust
0151 252 5153
m.w.beresford@liverpool.ac.uk



Thank you for reading this leaflet!

Appendix 3.3.7: Paediatric control patient/parental consent form

Parental Consent Form
(Paediatric Controls - Liverpool only)
UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 - 1 st January 2016) for the above study and have had the chance to ask questions.	
2.	I understand my child's taking part in voluntary and that I am free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected.	
3.	I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my child's blood may be used to investigate Lupus.	
5.	I agree that a small amount of my child's urine may be used to investigate the kidney.	
6.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
7.	I give permission for my GP to be informed that information about me is to be held on the study database.	
8.	I agree for my child to take part in the above study	

Name of patient

Name of person with parental
responsibility for patient

Date

Signature

Name of person taking consent
(if different from researcher)

Date

Signature

Researcher

Date

Signature

1 copy for patient and person with parental responsibility, 1 for researcher, 1 to be kept with hospital notes

Appendix 3.3.8: Paediatric control patient information sheet

Appendix 3.3.8.1 Paediatric control patient information sheet for under 6



**UK
Children's
Lupus Study**

Information sheet
for patients aged less than 6 years
(Paediatric controls - Liverpool only)

Note to reader: Please read the whole sheet to yourself before reading it to your child

Some children are sick because they have an illness called Lupus.

Doctors want to find out why some children get Lupus and others don't

To do this we need to compare children with Lupus to children without Lupus, like you

UK JSLC Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Why have I been chosen?

Because you do not have Lupus

What will happen if I say yes?

- We would like to collect information about how you are.
- We will take a small sample of your blood (2-3 teaspoons) when it is already being taken or you are going for an operation. There are NO extra needles!
- We will also ask for a urine sample.

Do I have to help?

No, not if you or your mum / dad or the grown up looking after you decide you do not want to. You will be looked after just the same.

Will it do some good if I say yes?

Yes, you will be helping us invent better treatments for sick children with Lupus

What do I have to do now?

We are asking you to say "Yes" or "No" to the question:
"Can we collect information about how you are and a small blood and urine sample?"

What if I have questions?

If you have any questions you or you mum or dad, or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!



Appendix 3.3.8.2: Paediatric control patients' information sheet for 6-12



UK Children's Lupus Study

Information sheet for
patients aged 6-12
years

(Paediatric controls - Liverpool only)

Why is this research being done?

This research looks at a condition called "Lupus". We want to understand more about what causes it and the best ways to treat it.

To do this we need to compare children with Lupus to children without Lupus.

Why have I been chosen?

You are being asked to take part in this study because you do not have Lupus.

Do I have to help?

No, not if you, your mum / dad or the grown up looking after you do not want to. You will be looked after just the same.

What will happen if I say yes?

You will be asked to write your name on a form. This is to say that you understand the study and what will happen. You will be given your own copy of the form to keep as well as this leaflet.

We will take a sample of your blood (2-3 teaspoons) when you are going for your operation. There are NO extra needles!

We will also ask if you can give us a urine sample. We use this to look at how the kidneys work in Lupus.

Will it do some good if I say yes?

Using your samples, we will work on ways to us create better treatments for children with Lupus.

Will anything bad happen to me if I take part?

No, nothing bad will happen to you.

What do I have to do now?

If you want to help, you and your mum or dad or the grown up looking after you will have to say it's ok.

What if I have questions?

If you have any questions you or you mum or dad or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!



Appendix 3.3.8.3: Paediatric control patient information sheet for 13-15



UK Children's Lupus Study

Information sheet for patients aged 13-15 years
(Paediatric controls - Liverpool only)

Why is this study being done?

This study is looking at a condition called "Lupus". We don't know a lot about what causes it or the best ways to treat it. We hope that by understanding more about what causes Lupus we can create better treatments.

To do this we need to compare children with Lupus to children without Lupus, like you.

Why have I been chosen?

You are being asked to take part in this study because you do not have Lupus.

Do I have to help?

No, it's up to you and your parents or the adult looking after you. You can change your mind at any time during the research without giving a reason. This will not affect the care you receive.

What will happen if I say yes?

- We will take a blood sample (2-3 teaspoons). If you need blood tests for another reason we will take it at the same time.
- We will also ask if you can give us a urine sample.
- We will collect information about your age, gender and your general health.

Will it do some good if I say yes?

By doing experiments on your samples, you will be helping us create better treatments for children with Lupus in the future.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small and you will not feel any different. Helping us with the study will not affect your hospital care.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Who is organising the research?

It is being organised by a group of doctors and nurses from around the country called the "UK Lupus Study Group". It is run from Alder Hey Hospital.

Has the study been checked?

The Liverpool Paediatric Research Ethics Committee has approved this study.

What do I have to do now?

If you are happy to take part you will be asked to write your name on a form. This is to say that you understand the study and what will happen. You will be given your own form to keep as well as this leaflet.

Who is organising the research?

It is being organised by a group of doctors and nurses from around the country called the "UK Lupus Study Group". It is run from Alder Hey Hospital.

What if I have questions?

If you have any questions you or your mum or dad or the grown up looking after you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford
Chief Investigator UK JSLE Study Group
Alder Hey Children's NHS Foundation Trust
0151 252 5153
m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!



UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Appendix 3.3.8.4: Paediatric control patient information sheet for 16 and over



**UK Children's
Lupus Study**

**Information sheet for patients aged 16
years and older**

(Paediatric controls - Liverpool only)

What is a study? Why is this being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body attacks itself. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare the blood cells of children with Lupus and without Lupus to see why Lupus cells are reacting against themselves. In addition we need to look at urine to detect inflammation of the kidney, a common complication of Lupus.

This project is part of a UK wide study of children with Lupus.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Why am I being asked to take part?

Because you do not have Lupus.

Do I have to take part?

No, it's up to you. You can change your mind at any point without giving a reason. This will not affect the care you receive.

What will happen if I say yes?

- We will take a sample of your blood (2-3 teaspoons). If you are having blood tests for another reason we will take it at the same time.
- We will also ask if you can give us a urine sample. We use this to look at how the kidneys work in Lupus.
- We will collect information about your age, gender and your general health.
- We will keep your name and hospital details on a list in your hospital's research office so that we know you are in the study. Your participation will be kept strictly confidential.
- We will tell your GP you are helping us with the study.

Will it do some good if I say yes?

By doing experiments on the samples given you will be helping us create better treatments for children with Lupus in the future.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small and you will not feel any different. Helping us with the study will not affect your hospital care.

What will happen to the results of the research study?

Everything we discover will be presented at scientific conferences and published in medical journals. You will not be identified in any way.

Who is organising the research?

It is being organised by a group of doctors and nurses from around the country called the "UK Lupus Study Group". It is run from Alder Hey Children's NHS Foundation Trust.

The research is being funded by Alder Hey Children's NHS Foundation Trust, its Research and Development Department at Alder Hey, the charity Lupus UK and The Alder Hey Renal Fund.

No one, including your doctor receives any payment for being involved in this study.

Has the study been checked?

The Liverpool Paediatric and Research Ethics Committee have approved this study.

What will happen to the information collected about me?

All information and samples collected from you will be strictly confidential and anonymised. This means that no-one will know it belongs to you.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Forms will be kept in your hospital's research office. They will be stored in locked filing cabinets at all times, in rooms that are locked when non-attended.

All information kept on study computers (in offices of the UK JSLE Study Group at the Institute Child Health, University of Liverpool) will only record data using your study number and be strictly confidential. Details identifying who you are will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.

If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford
Chief Investigator UK JSLE Study Group
Alder Hey Children's NHS Foundation Trust
0151 252 5153
m.w.beresford@liverpool.ac.uk



**Thank you for reading this
Leaflet!**

Appendix 3.3.10: Paediatric control patient consent form

Patient's Consent Form

(Paediatric Controls - Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 - 1 st January 2016) for the above study and have had the chance to ask questions.	
2.	I understand taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my blood may be used to investigate Lupus.	
5.	I agree that a small amount of my urine may be used to investigate the kidney.	
6.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
7.	I give permission for my GP to be informed that information about me is to be held on the study database.	
8.	I agree that I will take part in the above study.	

Name of patient

Date

Signature

Name of person taking consent
(if different from researcher)

Date

Signature

Researcher

Date

Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

Appendix 3.3.11: Adult volunteer information sheet



PARTICIPANT INFORMATION SHEET

- 1. Study Title:** Analysis of normal blood leukocyte function using cells from healthy volunteers.
- 2. Version Number and date.** [Version 3, January 2015](#)
- 3. Invitation.** You are being invited to participate in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and feel free to ask us if you would like more information or if there is anything that you do not understand. Please also feel free to discuss this with your friends, relatives and GP if you wish. We would like to stress that you do not have to accept this invitation and should only agree to take part if you want to.
- 4. What is the purpose of the study?** The purpose of the study is to derive baseline data for how specialised white blood cells in the blood, called leucocytes, behave in normal healthy donors. These cells normally help prevent infection as part of the normal immune response. This will involve measuring protein expression, cell function and/or analysis of genes related to immune function as part of a series of studies in the Dept of Women's and Children's Health, University of Liverpool.
- 5. Why have I been chosen?** You have been chosen because you are a normal healthy person who has indicated they are willing to give blood.
- 6. Do I have to take part?** No. It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason.
- 7. What will happen if I take part?** You will be asked to give 5-30ml blood. We may ask to take blood again, so that we can reproduce our results, but no more frequently than 10 times per year. However, we will only do this if you are well and completely happy to give the extra samples. You are free to say no and opt out of the study at any stage. Blood samples will be tested in the laboratory at the department of Women's and Children's health. All tests will be performed by the recognised researchers concerned with this project. Samples taken will not be identified by a person's name after collection but will be anonymised and only identified to the research worker by a code number. Samples will be tested immediately not stored in department refrigerators or freezers until tests are performed. Only the investigators will have access to this material. Samples will be destroyed after analysis and will not be used for any other research studies.
- 8. Expenses and payments.** We do not offer any payments for participants.

- 9. What are the risks in taking part?** There are no risks additional to those normally encountered when giving a blood sample for clinical purposes. You may experience slight bruising at the needle site.
- 10. Are there any benefits in taking part?** There are no benefits intended for participants but you will be contributing to research which may eventually lead to the development of improved therapies.
- 11. What if I am unhappy or if there is a problem?** If you are unhappy, or if there is a problem, please feel free to let us know by contacting Dr. Brian Flanagan (0151 282 4732) and we will try to help. If you remain unhappy or have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide them with name of this study and the researcher(s) involved, and the details of the complaint you wish to make.
- 12. Will my taking part in the study be kept confidential?** Yes, the results of the study will be anonymised and any information about you kept confidential. The data may be kept for up to five years on password protected computers.
- 13. What will happen to the results of this study?** The results of the research may ultimately be published in scientific papers but you will not be identified in any report.
- 14. What will happen if I want to stop taking part?** You are free to withdraw at anytime, without explanation. Results up to the period of withdrawal may be used, if you are happy for this to be done. Otherwise you may request that they are destroyed and no further use is made of them.
- 15. Who can I contact if I have further questions?** For any further questions or concerns about participating in this study please contact:

Dr Brian Flanagan
Department of Women's and Children's health,
University of Liverpool,
Alder hey Hospital,
Eaton Road,
Liverpool L12 2AP
Tel. 0151 282 4732
email fla1@liv.ac.uk

- 16. Duty of care to research participants.** Occasionally research studies can reveal significant unexpected abnormalities which require medical follow-up, either for further investigation or (more rarely) treatment. You will be asked to agree that if any significant abnormality is found, we will send the report to your GP, who will be able to take it further with you. Should this be needed the study PI will discuss this with you.

Thank you for taking the time reading this information sheet, please complete the attached consent form if you wish to participate.

Appendix 3.3.12: Adult volunteer consent form



Committee on Research Ethics

PARTICIPANT CONSENT FORM

Title of Research: Analysis of normal blood leukocyte function using cells
Project: from healthy volunteers.

Researcher(s): Dr BF Flanagan

Please
initial box

1. I confirm that I have read and have understood the information sheet [version 3 dated January 2015](#) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected.
3. Data will be stored electronically in a anonymised form and will not be identified using my name, only a sample code number. I understand that, under the Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish.
4. I understand that confidentiality and anonymity will be maintained and it will not be possible to identify me in any publications.
5. If an unexpected significant abnormality is discovered I consent to my GP being contacted
6. I agree to take part in the above study.

Participant Name	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

Principal Investigator:
Name: Dr BF Flanagan
Work Address: Department of Women's and Children's Health
 Univ. of Liverpool
Work Telephone: 0151 282 4752
Work Email: flb1@liv.ac.uk

Student Researcher:
Name:
Work Address:
Work Telephone:
Work Email:

Appendix B: Reagents

Appendix 1.1: Assay materials

Material – plastic ware	Supplier
1.5 ml tubes	Fisher scientific, UK
5 ml tubes	Fisher scientific, UK
10 ml tubes	Fisher scientific, UK
25 ml universals	Fisher scientific, UK
50 ml falcon	Starlab, UK
5, 10, 25 ml stripette	Fisher scientific, UK
Transfer pipette PE	Fisher scientific, UK
Tips (10, 20, 200, 1000)	Starlab/ Fisher scientific, UK
24-well plates	Fisher scientific, UK
96-well round bottom plates	Fisher scientific, UK
Corning™ Clear Polystyrene 96-Well Microplates	Fisher scientific, UK
Cover Slips 13mm diameter	Appleton Woods
Microscope slide	Fisher scientific, UK
Test tube 12x75mm	Elkay, UK
Syringe-drive filter unit, 0.22 µm	Millex-GP, UK

Appendix - Table 1: Plasticware used for experiments.

Material – sample collection	Supplier
Micro tube 1.3ml Z	Sarstedt, Germany
Lithium heparin vacutainer	Sarstedt, Germany
Scalp vein set	Nipro, UK
10 ml, 20 ml Syringe	BD Plastipak, UK

Appendix - Table 2: Materials used for collection of blood.

Material – Cell isolation and incubations	Supplier
HetaSep	Stemcell, UK
PBS	Fisher, UK
Roswell Park Memorial Institute 1640 (RPMI-1640) with L-Glutamine	Lonza, Belgium
Histopaque-1077	Sigma-Aldrich, UK
Ammonium chloride	Sigma-Aldrich, UK
EDTA	Fisher, UK
Potassium Bicarbonate	Sigma-Aldrich, UK
EasySep™ Buffer	Stemcell, UK
EasySep™ Human Neutrophil Enrichment Kit	Stemcell, UK
<ul style="list-style-type: none"> - EasySep™ Human Neutrophil Enrichment Cocktail - EasySep™ Magnetic Particles 	
Foetal bovine serum (FBS)	Life Technologies, UK
Recombinant GM-CSF	R&D Systems, UK
Recombinant IFNα	PeptoTech, UK
Recombinant TNFα	PeptoTech, UK

Appendix - Table 3: Materials used for cell isolation and stimulation.

Material – real-time PCR	Supplier
TRIzol	Life technologies (Molecular probes), UK
RNeasy Mini Kit	Qiagen, Germany
<ul style="list-style-type: none"> - RNeasy Mini Spin Columns - Collection Tubes (1.5 ml) - Collection Tubes (2 ml) - Buffer RLT - Buffer RW1 - Buffer RPE - RNase-Free Water 	
Chloroform	Sigma-Aldrich, UK
Isopropanol	Sigma-Aldrich, UK
Ethanol	University of Liverpool
RNase-free DNase set	Qiagen, Germany
<ul style="list-style-type: none"> - Lyophilized DNase - Nuclease-free water - RDD-Buffer 	
AffinityScript QPCR cDNA Synthesis Kit	Agilent Technologies, USA
<ul style="list-style-type: none"> - 10X AffinityScript RT buffer - dNTP mix - RNase block RNase inhibitor - AffinityScript RTase 	
Brilliant II SYBR® Green QPCR Master Mix	Agilent Technologies, USA
<ul style="list-style-type: none"> - 2× Brilliant II SYBR® Green QPCR Master Mix - ROX dye, 1 mM 	
Precision™ Reverse Transcription Premix 2	Primerdesign, UK
RT negative control premix	Primerdesign, UK
Precision®PLUS qPCR Master Mix for the Stratagene with SYBR-Green	Primerdesign, UK
Optical Tube and Cap 8xstrip	Agilent Technologies, USA
BrightWhite qPCR Plates	Primerdesign, UK

Appendix - Table4: Materials used for real-time PCR.

Material – Flow cytometry	Supplier
Annexin V-FITC Apoptosis Detection	Sigma, USA
<ul style="list-style-type: none"> - Annexin V-FITC Conjugate - Propidium Iodide Solution - 10x Binding buffer 	
Paraformaldehyde 37%	Sigma-Aldrich, UK
Triton-X 100	BDH Limited Poole England, UK

Appendix - Table 5: Materials used in flow cytometric assays.

Materials – phagocytosis assay	Supplier
pHrodo™ Red <i>S. aureus</i> BioParticles® Conjugate	Life technologies (Molecular probes), UK
pHrodo™ Red <i>E.coli</i> BioParticles® Conjugate	Life technologies (Molecular probes), UK
pHrodo™ Red Zymosan A BioParticles® Conjugate	Life technologies (Molecular probes), UK
HBSS	Sigma-Aldrich, UK
HEPES	BDH Limited Poole England, UK
Tris-Base	Fisher scientific, UK
NaCl	Fisher scientific, UK
ProLong™ Gold Antifade Mountant with DAPI	Invitrogen, Fisher Scientific, USA

Appendix - Table 6: Materials used to investigate phagocytosis.

Materials - whole blood assay	Supplier
2.0ml Graduated, Skirted Tube, Natural EasyGrip Cap (Sterile)	Starlab, UK
Native nucleosome (HEK293-derived)	AMS Biotechnology, UK
SigmaUltra Sucrose	Sigma-Aldrich, UK
Chicken Blood Cells (Adult) in Alsever's	TCS Biosciences Ltd, UK
BD FACS™ Lysing Solution	BD Biosciences, UK
Brefeldin A Solution 1000x	Invitrogen, Fisher Scientific, USA

Appendix - Table 7: Materials used for whole blood assays.

Materials ELISA	Supplier
ELISA kit FCGR3b <ul style="list-style-type: none"> - FCGR3B Microplate 96 Wells - FCGR3B Lyophilized Standard - 100X Biotinylated FCGR3B Detector Antibody - 100X Avidin-HRP Conjugate - Sample Diluent - Detector Antibody Diluent - Conjugate Diluent - 25X Wash Buffer - Stop Solution - TMB Substrate 	Aviva Systems Biology, USA
DuoSet® ELISA human S100A9, S100A8/S100A9 <ul style="list-style-type: none"> - Capture Antibody - Human S100A9, S100A8/S100A9 Standard - Detection Antibody - Streptavidin-HRP 	R&D systems, UK
BSA	Sigma-Aldrich, UK
TMB Substrate Reagent Set <ul style="list-style-type: none"> - Hydrogen peroxide (H₂O₂) - 3,3', 5,5' tetramethylbenzidine (Substrate B) 	BD Biosciences, UK
H₂SO₄	BDH Limited Poole England, UK

Appendix - Table 8: Materials used for ELISAs.

Appendix 1.2: List of antibodies

All antibodies were mouse anti-human antibodies, supplied by BD Biosciences, UK if not stated otherwise.

Antigen	Dye	Fc-part	Dilution
TLR2	PE	IgG1, κ	1:40
CD16b	FITC	IgG2a, κ	1:10
Isotype	FITC	IgG2a, κ	1:10
Isotype (extracellular)	PE	IgG1, κ	1:80
S100A9	PE	IgG1, κ	1:20
Isotype (intracellular)	PE	IgG1, κ	1:80

Appendix - Table 9: Antibodies used for flow cytometry and immunohistochemistry.

Appendix C: Metabolite profiles

Appendix 1.1: Pattern file for serum referenced to glucose

PATTERN	=	1 human			
serum					
GROUP	=				
DESCRIPTION	=	serum pattern file glucose referenced			
AUTHOR	=	Anna			
DIM	=	2			
ORIGIN	=	1			
ITEMS	=	279			
0	0	8.4644	8.4569	0	Formate.1
0	0	8.2378	8.2327	0	UNID.2
0	0	8.2077	8.183	0	Hypoxanthine.3
0	0	7.9092	7.8767	0	UNID.4
0	0	7.8767	7.843	0	UNID.5
0	0	7.843	7.8276	0	UNID.6
0	0	7.8276	7.7812	0	Histidine UNID.7
0	0	7.7812	7.7607	0	UNID.8
0	0	7.7607	7.7467	0	UNID.9
0	0	7.7033	7.6808	0	UNID.10
0	0	7.6808	7.6678	0	Tau-Methylhistidine.11
0	0	7.446	7.4066	0	Phenylalanine.12
0	0	7.3897	7.3809	0	UNID.13
0	0	7.3809	7.3693	0	N-Acetylserotonin Phenylalanine.14
0	0	7.3693	7.3552	0	Phenylalanine N-Acetylserotonin.15
0	0	7.3552	7.3439	0	UNID.16
0	0	7.3439	7.3141	0	Phenylalanine UNID.17
0	0	7.296	7.2821	0	UNID.18
0	0	7.2699	7.2558	0	UNID.19
0	0	7.2558	7.2405	0	UNID.20
0	0	7.2405	7.226	0	UNID.21
0	0	7.226	7.213	0	UNID.22
0	0	7.2106	7.1808	0	Tyrosine.23
0	0	7.1802	7.1713	0	UNID.24
0	0	7.1622	7.1509	0	UNID.25
0	0	7.1509	7.1391	0	UNID.26
0	0	7.1391	7.1302	0	UNID.27
0	0	7.0788	7.0629	0	UNID Histidine.28
0	0	7.0553	7.0222	0	UNID.29
0	0	7.0222	7.0047	0	UNID.30
0	0	7.0047	6.9961	0	UNID.31
0	0	6.9961	6.9862	0	UNID.32
0	0	6.9765	6.9601	0	UNID.33
0	0	6.9601	6.9431	0	UNID.34

0	0	6.9291	6.9183	0 UNID.35
0	0	6.9273	6.8928	0 Tyrosine.36
0	0	6.8928	6.886	0 UNID.37
0	0	6.7512	6.7399	0 UNID.38
0	0	6.7399	6.7326	0 UNID.39
0	0	5.387	5.3792	0 UNID.40
0	0	5.3792	5.373	0 UNID.41
0	0	5.3636	5.2486	0 mobile_unsaturated_lipids.42
0	0	5.2486	5.2295	0 Glucose.43
0	0	5.2203	5.2136	0 UNID.44
0	0	5.2136	5.2037	0 UNID.45
0	0	5.2037	5.1948	0 UNID.46
0	0	5.1948	5.1845	0 UNID Mannose.47
0	0	4.9089	4.8994	0 Mannose.48
0	0	4.4795	4.4299	0 UNID.49
0	0	4.3849	4.3699	0 UNID.50
0	0	4.3699	4.3614	0 UNID.51
0	0	4.2937	4.286	0 UNID.52
0	0	4.286	4.2798	0 UNID.53
0	0	4.2798	4.2719	0 UNID.54
0	0	4.2719	4.2662	0 UNID.55
0	0	4.2662	4.2594	0 UNID.56
0	0	4.2571	4.2526	0 Threonine.57
0	0	4.2526	4.2486	0 Threonine.58
0	0	4.2455	4.2413	0 Threonine.59
0	0	4.1708	4.1657	0 UNID.60
0	0	4.1614	4.1529	0 UNID.61
0	0	4.1529	4.143	0 UNID.62
0	0	4.1376	4.1339	0 UNID.63
0	0	4.1344	4.094	0 Lactate.64
0	0	4.094	4.0855	0 UNID.65
0	0	4.0855	4.0776	0 UNID.66
0	0	4.0776	4.0739	0 UNID.67
0	0	4.0739	4.0671	0 UNID.68
0	0	4.066	4.0603	0 UNID myo-Inositol.69
0	0	4.0586	4.0504	0 Creatinine.70
0	0	4.0139	4.0075	0 UNID.71
0	0	4.0075	4.0007	0 UNID Arabinose.72
0	0	4.0007	3.9952	0 UNID Histidine.73
0	0	3.9925	3.9866	0 UNID.74
0	0	3.9866	3.9811	0 UNID Histidine.75
0	0	3.9811	3.9761	0 UNID Histidine.76
0	0	3.9738	3.9665	0 UNID.77
0	0	3.9665	3.9601	0 UNID.78
0	0	3.9574	3.951	0 Creatine_phosphate.79

0	0	3.9401	3.9359	0 UNID.80
0	0	3.9359	3.9318	0 UNID Mannose.81
0	0	3.9318	3.9255	0 Creatine Mannose.82
0	0	3.9203	3.887	0 Glucose.83
0	0	3.8816	3.8715	0 UNID.84
0	0	3.8718	3.8663	0 UNID.85
0	0	3.8636	3.853	0 Glucose.86
0	0	3.8517	3.8466	0 UNID.87
0	0	3.847	3.8143	0 Glucose.88
0	0	3.8124	3.8096	0 UNID.89
0	0	3.8096	3.8067	0 UNID.90
0	0	3.8058	3.8016	0 N-Nitrosodimethylamine.91
0	0	3.8018	3.798	0 UNID.92
0	0	3.7985	3.7944	0 Alanine.93
0	0	3.7943	3.7904	0 Guanidoacetate Arginine.94
0	0	3.7903	3.7804	0 Glucose Glycerol.95
0	0	3.7804	3.7731	0 Glucose Glycerol.96
0	0	3.7731	3.7711	0 UNID.97
0	0	3.7711	3.7343	0 Glucose.98
0	0	3.7332	3.731	0 UNID.99
0	0	3.7302	3.715	0 Glucose.100
0	0	3.7165	3.7113	0 UNID.101
0	0	3.7108	3.7034	0 Glucose.102
0	0	3.6925	3.687	0 UNID.103
0	0	3.6793	3.6757	0 UNID.104
0	0	3.6757	3.6707	0 Glycerol.105
0	0	3.6685	3.6623	0 UNID.106
0	0	3.6563	3.6511	0 UNID.107
0	0	3.6501	3.6448	0 Glycerol.108
0	0	3.6448	3.6403	0 UNID.109
0	0	3.6352	3.6295	0 UNID.110
0	0	3.6224	3.6185	0 myo-Inositol.111
0	0	3.6185	3.6137	0 Valine.112
0	0	3.6137	3.606	0 Valine.113
0	0	3.6063	3.6037	0 UNID.114
0	0	3.6037	3.5998	0 UNID.115
0	0	3.5975	3.5941	0 UNID.116
0	0	3.5941	3.5884	0 Threonine.117
0	0	3.5884	3.5833	0 Glycerol.118
0	0	3.5833	3.5797	0 UNID.119
0	0	3.5797	3.576	0 UNID.120
0	0	3.576	3.5712	0 Glycerol.121
0	0	3.5689	3.5659	0 Glycine Glycerol.122
0	0	3.5569	3.5513	0 Glucose Glycerol.123
0	0	3.5513	3.4506	0 Glucose.124

0	0	3.4501	3.4447	0 Acetoacetate UNID.125
0	0	3.4447	3.4414	0 UNID.126
0	0	3.4407	3.4255	0 Glucose.127
0	0	3.4255	3.4171	0 Glucose UNID.128
0	0	3.4154	3.3941	0 Glucose.129
0	0	3.3884	3.384	0 UNID.130
0	0	3.384	3.3783	0 UNID.131
0	0	3.3783	3.374	0 UNID.132
0	0	3.368	3.3616	0 Methanol.133
0	0	3.3616	3.3576	0 UNID.134
0	0	3.3576	3.3528	0 UNID.135
0	0	3.3456	3.3398	0 UNID.136
0	0	3.3369	3.3318	0 UNID.137
0	0	3.3256	3.3188	0 UNID.138
0	0	3.3086	3.3041	0 myo-Inositol.139
0	0	3.2985	3.2936	0 UNID Histamine.140
0	0	3.2936	3.2877	0 myo-Inositol.141
0	0	3.2809	3.2746	0 UNID.142
0	0	3.2746	3.2695	0 UNID.143
0	0	3.2695	3.2653	0 Glucose.144
0	0	3.2653	3.2608	0 Betaine Glucose.145
0	0	3.2598	3.2494	0 Trimethylamine_N-oxide Glucose.146
0	0	3.2483	3.2443	0 UNID Arginine.147
0	0	3.2415	3.2355	0 Glucose.148
0	0	3.2282	3.2214	0 UNID.149
0	0	3.2214	3.1953	0 Mobile-N(CH3)3.150
0	0	3.1953	3.1865	0 Mobile-N(CH3)3 glycerol- phosphocholine.151
0	0	3.1732	3.1676	0 UNID.152
0	0	3.1571	3.1528	0 N-Nitrosodimethylamine.153
0	0	3.1528	3.1373	0 UNID Histidine.154
0	0	3.133	3.1265	0 UNID Histidine.155
0	0	3.1183	3.1112	0 UNID Histidine.156
0	0	3.1112	3.1038	0 UNID.157
0	0	3.0769	3.0713	0 Ornithine.158
0	0	3.0713	3.0662	0 UNID.159
0	0	3.0636	3.0571	0 Ornithine.160
0	0	3.0506	3.0427	0 Creatinine Lysine Creatine_phosphate.161
0	0	3.0427	3.0373	0 Creatine.162
0	0	3.0373	3.0291	0 Lysine.163
0	0	3.0291	3.0152	0 UNID Lysine.164
0	0	3.0152	3.0024	0 UNID.165
0	0	3.0024	2.9917	0 UNID.166
0	0	2.9707	2.9625	0 UNID.167
0	0	2.9625	2.9552	0 UNID.168

0	0	2.9416	2.935	0 Asparagine.169
0	0	2.9336	2.9305	0 Asparagine.170
0	0	2.9305	2.9248	0 UNID.171
0	0	2.9186	2.9064	0 UNID.172
0	0	2.8923	2.8869	0 UNID.173
0	0	2.8713	2.8645	0 Asparagine.174
0	0	2.8645	2.8597	0 UNID.175
0	0	2.858	2.8538	0 Asparagine.176
0	0	2.845	2.8385	0 UNID.177
0	0	2.8051	2.712	0 Polyunsaturated_fatty_acids.178
0	0	2.6898	2.6813	0 Citrate.179
0	0	2.6629	2.6564	0 Citrate.180
0	0	2.6517	2.6453	0 Methionine.181
0	0	2.6385	2.6325	0 Methionine.182
0	0	2.553	2.545	0 Citrate.183
0	0	2.5377	2.5295	0 Isocitrate.184
0	0	2.5258	2.5197	0 Citrate.185
0	0	2.5111	2.5054	0 UNID.186
0	0	2.5054	2.4997	0 UNID Glutamine.187
0	0	2.4955	2.4847	0 UNID Glutamine.188
0	0	2.4815	2.4327	0 Glutamine.189
0	0	2.4327	2.4263	0 UNID.190
0	0	2.4263	2.4219	0 UNID.191
0	0	2.4219	2.4163	0 UNID.192
0	0	2.4163	2.4111	0 Succinylacetone.193
0	0	2.4082	2.4017	0 Succinylacetone UNID.194
0	0	2.4017	2.3969	0 UNID.195
0	0	2.3969	2.391	0 UNID.196
0	0	2.3839	2.3762	0 UNID.197
0	0	2.3762	2.3694	0 Pyruvate Glutamate.198
0	0	2.3694	2.3655	0 UNID.199
0	0	2.3626	2.339	0 UNID Glutamate.200
0	0	2.3339	2.3299	0 UNID.201
0	0	2.3303	2.3268	0 UNID.202
0	0	2.3193	2.3139	0 UNID.203
0	0	2.3136	2.3091	0 UNID.204
0	0	2.3091	2.3084	0 UNID.205
0	0	2.3084	2.2979	0 UNID.206
0	0	2.2979	2.2934	0 UNID.207
0	0	2.2934	2.2893	0 UNID.208
0	0	2.2851	2.2774	0 Acetoacetate.209
0	0	2.2774	2.2725	0 UNID Succinylacetone.210
0	0	2.2725	2.2707	0 UNID Valine.211
0	0	2.2703	2.2647	0 UNID Valine.212
0	0	2.266	2.2615	0 UNID Valine.213

0	0	2.2615	2.225	0 LIPID_hOOC-CH2 Acetone.214
0	0	2.1862	2.1811	0 UNID.215
0	0	2.176	2.172	0 UNID.216
0	0	2.172	2.1689	0 UNID.217
0	0	2.1678	2.163	0 UNID.218
0	0	2.163	2.1567	0 Glutamine UNID.219
0	0	2.1533	2.1463	0 Glutamine O-Acetylcholine.220
0	0	2.1463	2.14	0 UNID Glutamine.221
0	0	2.14	2.1352	0 Glutamine.222
0	0	2.1352	2.1083	0 UNID Glutamine Methionine.223
0	0	2.1083	2.1041	0 UNID.224
0	0	2.1041	2.099	0 UNID.225
0	0	2.099	2.0953	0 UNID.226
0	0	2.0933	2.0621	0 UNID.227
0	0	2.0607	2.0296	0 Glycoprotein.228
0	0	2.0296	1.9997	0 LIPID_FA_=Ch-CH2-CH2-.229
0	0	1.9341	1.9285	0 Arginine Lysine.230
0	0	1.9219	1.9166	0 Acetate.231
0	0	1.9166	1.912	0 Arginine.232
0	0	1.9081	1.9001	0 Arginine Lysine N-Acetylserotonin.233
0	0	1.8976	1.8914	0 Lysine.234
0	0	1.8894	1.8831	0 Lysine.235
0	0	1.7616	1.7557	0 UNID.236
0	0	1.7557	1.7518	0 UNID.237
0	0	1.7518	1.6666	0 Leucine Arginine Lysine.238
0	0	1.6666	1.661	0 UNID.239
0	0	1.661	1.6559	0 UNID.240
0	0	1.6502	1.643	0 UNID.241
0	0	1.6381	1.6328	0 UNID.242
0	0	1.6171	1.5335	0 Lipid_dolichol.243
0	0	1.4932	1.4708	0 Alanine.244
0	0	1.4429	1.4377	0 UNID.245
0	0	1.4377	1.4339	0 UNID.246
0	0	1.4303	1.4254	0 UNID.247
0	0	1.4254	1.417	0 UNID.248
0	0	1.4118	1.408	0 UNID.249
0	0	1.408	1.4023	0 UNID.250
0	0	1.3977	1.3913	0 UNID.251
0	0	1.3389	1.3169	0 Lactate.252
0	0	1.3166	1.2504	0 mobile_lipid.253
0	0	1.2504	1.2453	0 UNID.254
0	0	1.2412	1.2373	0 UNID.255
0	0	1.2063	1.2024	0 UNID.256
0	0	1.1997	1.1799	0 Ethanol.257
0	0	1.1799	1.1752	0 UNID.258

0	0	1.1752	1.1696	0 Ethanol.259
0	0	1.1696	1.1647	0 UNID.260
0	0	1.1516	1.1367	0 Propylene_glycol.261
0	0	1.0908	1.0872	0 UNID.262
0	0	1.0806	1.0749	0 Methylsuccinate UNID.263
0	0	1.0677	1.0636	0 Methylsuccinate.264
0	0	1.0508	1.0321	0 Valine.265
0	0	1.0174	1.0007	0 Isoleucine.266
0	0	1.0007	0.9836	0 Valine.267
0	0	0.9836	0.9794	0 UNID.268
0	0	0.9743	0.9666	0 Leucine.269
0	0	0.9666	0.9571	0 Leucine UNID.270
0	0	0.9571	0.9528	0 UNID.271
0	0	0.9528	0.9474	0 Leucine Isoleucine.272
0	0	0.941	0.9345	0 UNID Isoleucine.273
0	0	0.9281	0.9235	0 UNID Isoleucine.274
0	0	0.9203	0.9074	0 UNID.275
0	0	0.8995	0.786	0 LDL-mobile_lipid-CH3.276
0	0	0.7112	0.6898	0 UNID.277
0	0	0.6768	0.646	0 UNID.278
0	0	0.1641	0.1591	0 UNID.279

Appendix - Table 10: Pattern file for serum referenced to glucose.

Appendix 1.2: Confidence table of serum metabolites

METABOLITE	TOTAL ASSIGNED FEATURES	OVERLAPPED	NON-OVERLAPPED	NOTED PEAKS PER FEATURE	ASSIGNMENT CONFIDENCE
ACETATE	1	0	1	1	low
ACETOACETATE	2	1	1	1	low
ALANINE	2	1	1	4 (quadruplet), 2 (doublet)	medium
ARGININE	6	6	0	3 (triplet), 3 (triplet), multiplets	low
ASPARAGINE	4	2	1	4 (quadruplet)	medium
BETAINE	2	2		1	low
CITRATE	4	2	2	1 (parts of two doublets)	high
CREATINE PHOSPHATE	2	2		1	low
CREATINE	2	2		1	low
CREATININE	2	1	1	1	low
FORMATE	1	0	1	1	low
GLUCOSE	17	8	9	2 (doublet), 2 (quadruplet), 1 (triplet), 2 (triplet), 4 (quadruplet), 2 (multiplet), multiplet	high
GLUTAMATE	2	2		multiplet	low
GLUTAMINE	8	6	2	Triplet, 1 (multiplet), multiplets	high
O-ACETYLCHOLINE	3	3		1, multiplet	low
GLYCEROL	7	5	2	1 (quadruplet), multiplet	high
GLYCINE	1	1		singlet	low
GLYCOPROTEIN	1	1		singlet	low
GUANIDOACETATE	1	1		singlet	low
HISTIDINE	9	9		singlet, 1 (quadruplet), doublet, 2 (quadruplet), multiplet	medium

ARABINOSE	2	2		1 (multiplet)	low
HYPOXANTHINE	1	0	1	2 (two singlets)	high
ISOCITRATE	3	2	1	doublet, quadruplet, multiplet	low
ISOLEUCINE	4	3	1	doublet, 1 (triplet)	medium
LACTATE	2		2	doublet, quadruplet	high
LDL-MOBILE_LIPID-CH3	1		1	multiplet (distinct feature)	medium
LEUCINE	4	4		1 (doublet), multiplet	medium
LIPID_DOLICHOL	1		1	multiplet (distinct feature)	medium
LIPID_FA_=CH-CH2-CH2-	1		1	multiplet (distinct feature)	medium
LIPID_HOOC-CH2	1	1		multiplet (distinct feature)	medium
LYSINE	7	7		1 (triplet), multiplet	medium
MANNOSE	4	3	1	2 (doublet), multiplet	medium
METHANOL	1	1		singlet	low
METHIONINE	3	2	1	1 (triplet), singlet	medium
METHYLSUCCINATE	2	1	1	1 (doublet), multiplet	medium
MOBILE_LIPID	1	1		multiplet (distinct feature)	medium
MOBILE_UNSATURATED_LIPIDS	1	1		multiplet (distinct feature)	medium
MOBILE-N(CH3)3	2	2		multiplet (distinct feature), singlet (distinct feature)	medium
GLYCEROL-PHOSPHOCHOLINE	1	1		singlet (distinct feature)	medium
MYO-INOSITOL	4	4		3 (triplet), 1 (triplet)	medium
N-ACETYLSEROTONIN	3	3		Singlet, 1 (doublet)	low
N-NITROSODIMETHYLAMINE	2	2		singlet	low
ORNITHINE	2	2		1 (triplet)	low
PHENYLALANINE	4	3	1	multiplet	high
POLYUNSATURATED_FATTY_ACIDS	1	1		multiplet (distinct feature)	medium
PROPYLENE_GLYCOL	1		1	doublet	
PYRUVATE	1	1		singlet	low
SUCCINYLACETONE	3	3		Singlet, 1 (triplet)	low

TAU-METHYLHISTIDINE	1		1	singlet	low
THREONINE	4	2	2	1 (doublet), 1 (multiplet)	medium
TRIMETHYLAMINE N-OXIDE	1	1		singlet	low
TYROSINE	2		2	multiplet	high
VALINE	7	3	4	Doublet, 1 (doublet), multiplet	high

Appendix - Table 11: Confidence table of assigned serum metabolites detected with ¹H NMR spectroscopy. Metabolites were considered part of a peak if the Chenomx Profiler® software detected more than 10% contribution to a peak. For each metabolite the number of features is listed which can be a singlet, doublet, triplet, quadruplet or a multiplet. Column three and four indicate the number of overlapped and non-overlapped peaks. Confidence was determined using the number of features and overlaps. Low= one singlet, or several features, but all overlapping; medium= very high number of features or at least one feature not overlapped; high= several features not-overlapped or one feature not-overlapped if in a very distinct region of the ppm scale/very distinct feature.

Appendix 1.3: Confidence table of urine metabolites

METABOLITE	TOTAL ASSIGNED FEATURES	OVERLAPPED	NON-OVERLAPPED	TYPE OF FEATURES	ASSIGNMENT CONFIDENCE
1-METHYLNICOTINAMIDE	5	2	3	2xsinglet, 2x doublets, 1 multiplet	high
1,3-DIMETHYLURATE	2	1	1	2xsinglet	medium
1,7-DIMETHYLXANTHINE	3	3	0	3xsinglet	low
2-AMINOADIPATE	6	6	0	1xquadruplet, 5x multiplet	low
2-FUROYLGLYCINE	5	5	0	2xdoublet, 1x quadruplet, 2xmultiplet	low
2-HYDROXYBUTYRATE	4	4	0	1xtriplet, 1x quadruplet, 2xmultiplet	low
2-HYDROXYISOBUTYRATE	1	0	1	singlet	low
2-METHYLGLUTARATE	5	4	1	1xdoublet, 1xtriplet, 3xmultiplet	medium
2-OXOGLUTARATE	2	2	0	2xtriplet	low
2'-DEOXYADENOSINE	9	9	0	2xsinglet, 1xtriplet, 3xquadruplet, 3xmultiplet	low
2'-DEOXYGUANOSINE	8	8	0	1xsinglet, 1xtriplet, 2xquadruplet, 4xmultiplet	low
2'-DEOXYINOSINE	9	9	0	2xsinglet, 1xtriplet, 2xquadruplet, 4xmultiplet	low
3-CHLOROTYROSINE	6	6	0	2xdoublet, 4x quadruplet	low
3-HYDROXY-3-METHYLGLUTARATE	3	3	0	1xsinglet, 2xdoublet	low
3-HYDROXYISOVALERATE	2	0	2	2xsinglet	low
3-HYDROXYMANDELATE	5	5	0	1xsinglet, 2xtriplet, 2xmultiplet	low
3-INDOXYLSULFATE	6	6	0	1xsinglet, 2xdoublet, 3xmultiplet	low
3-METHYLXANTHINE	2	2	0	2xsinglet	low
3-PHENYLLACTATE	6	6	0	3xquadruplet, 3xmultiplet	low
3-PHENYLPROPIONATE	7	7	0	4xquadruplet, 3xmultiplet	low

3,4-DIHYDROXYBENZENEACETATE	5	5	0	2xsinglet, 2xdoublets, 1xquadruplet	low
3,5-DIBROMOTYROSINE	4	4	0	singlet, 3xquadruplet	low
4-GUANIDINOBUTANOATE	6	6	0	4xtriplet, 2xquadruplet	low
4-HYDROXY-3-METHOXYMANDELATE	5	4	1	2xsinglet2xdoublet, 1x quadruplet	medium
4-HYDROXYBENZOATE	2	0	2	2xmultiplet	high
4-HYDROXYPHENYLACETATE	3	2	1	1xsinglet, 2xmultiplets	medium
4-PYRIDOXATE	3	3	0	3xsinglet	low
5-AMINOLEVULINATE	5	5	0	1xsinglet, 4xtriplet	low
5-HYDROXYINDOLE-3-ACETATE	5	5	0	1xsinglet, 2xdoublet, 1xquadruplet, 1xmultiplet	low
5-HYDROXYTRYPTOPHAN	8	8	0	1xsinglet, 2xdoublet, 4xquadruplet, 1xmultiplet	low
5-METHOXYSALICYLATE	4	4	0	1xsinglet, 1xdoublet, 1xquadruplet,	low
ACETATE	1	1	0	singlet	low
ACETOACETATE	2	2	0	singlet	low
ADENOSINE	8	6	2	singlet, 1xdoublet, 1xtriplet, 3xquadruplet	high
ADIPATE	2	2	0	2xmultiplets	low
ADP	8	8	0	2xsinglet, 1xdoublet, 2xtriplet, 3xmultiplet	low
ALANINE	2	1	1	1xdoublet, 1xquadruplet	medium
ALLANTOIN	4	4	0	4xsinglet	low
ANSERINE	11	11	0	3xsinglet, 1xdoublet, 2xquadruplet, 5xmultiplet	low
ARABINOSE	24	24	0	4xdoublet, 2xtriplet, 14xquadruplet, 4xmultiplet	low
ATP	8	8	0	2xsinglet, 1xdoublet, 2xtriplet, 3xmultiplet	low
BETAINE	2	1	1	2xsinglet	medium
CARNITINE	6	6	0	1xsinglet, 2xquadruplet, 3xmultiplet	low

CARNOSINE	10	10	0	3xsinglet, 2xquadruplet, 5xmultiplier	low
CELLOBIOSE	28	28	0	4xdoublet, 8xtriplet, 11xquadruplet, 5xmultiplier	low
CHOLINE	3	3	0	1xsinglet, 2xmultiplier	low
CIS-ACONITATE	2	1	1	1xdoublet, 1xmultiplier	medium
CITRATE	2	0	2	2xdoublet	high
CREATINE	2	1	1	2xsinglet	medium
CREATINE PHOSPHATE	2	2	0	2xsinglet	low
CREATININE	2	0	2	2xsinglet	high
DIMETHYLAMINE	1	0	1	1xsinglet	low
DTPP	9	9	0	1xtriplet, 8xmultiplier	low
ERYTHRITOL	6	6	0	2xquadruplet, 4xmultiplier	low
ETHANOLAMINE	2	2	0	2xmultiplier	low
FORMATE	1	1	0	singlet	low
FRUCTOSE	14	14	0	5xdoublet, 7xquadruplet, 2xmultiplier	low
FUCOSE	24	23	1	8xdoublet, 2xtriplet, 7xquadruplet, 7xmultiplier	medium
FUMARATE	1	1	0	singlet	low
GALACTARATE	2	2	0	2xsinglet	low
GALACTITOL	3	3	0	1xsinglet, 1xtriplet, 1xmultiplier	low
GALACTONATE	6	6	0	1xdoublet, 3xquadruplet, 2xmultiplier	low
GALACTOSE	14	14	0	2xdoublet, 9xquadruplet, 3xmultiplier	low
GALLATE	2	2	0	2xsinglet	low
GLUCARATE	4	4	0	2xdoublet, 1xtriplet, 1xquadruplet	low
GLUCONATE	6	6	0	1xdoublet, 1xtriplet, 4xmultiplier	low
GLUCOSE	14	14	0	2xdoublet, 3xtriplet, 7xquadruplet, 2xmultiplier	low
GLUCOSE-6-PHOSPHATE	14	14	0	2xdoublet, 4xtriplet, 5xquadruplet, 3xmultiplier	low
GLUCURONATE	10	9	1	4xdoublet, 4xtriplet, 1xquadruplet,	medium

				1xmultiplet	
GLUTAMINE	7	7	0	1xsinglet, 1xtriplet, 5xmultiplet	low
GLUTARIC ACID MONOMETHYL ESTER	4	4	0	1xsinglet, 2xtriplet, 1xmultiplet	low
GLYCINE	1	0	1	1xsinglet	low
GLYCOLATE	1	1	0	1xsinglet	low
GLYCYLPROLINE	17	17	0	1xsinglet, 2xdoublet, 2xquadruplet, 12xmultiplet	low
GUANIDOACETATE	1	1	0	singlet	low
HIPPURATE	5	2	3	1xsinglet, 1xdoublet, 3xmultiplet	high
HISTAMINE	4	4	0	1xsinglet, 1xdoublet, 2xtriplet	low
HISTIDINE	5	4	1	1xsinglet, 1xmultiplet, 3xquadruplet	medium
HOMOVANILLATE	5	4	1	2xsinglets, 2xdoublets, 1xquadruplet	medium
HYPOXANTHINE	2	1	1	2xsinglet	medium
IMIDAZOLE	2	2	0	2xsinglet	low
IMP	7	5	2	2xsinglet, 1xdoublet, 1xquadruplet, 3multiplet	high
INDOLE-3-ACETATE	7	7	0	2xsinglet, 2xdoublet, 3xmultiplet	low
INOSINE	8	8	0	2xsinglet, 1xdoublet, 1xtriplet, 4xquadruplet	low
ISOBUTYRATE	2	1	1	1xdoublet, 1xmultiplet	medium
KYNURENATE	5	5	0	1xsinglet, 1xdouplet, 1xtriplet, 2xmultiplet	low
KYNURENINE	6	6	0	1xdoublet, 1xtriplet, 2xquadruplet, 2xmultiplet	low
LACTATE	2	1	1	1xdoublet, 1xquadruplet	medium
LACTOSE	28	17	1	5xdoublet, 4xtriplet 16xquadruplet, 3xmultiplet	medium
LACTULOSE	42	41	1	2xsinglet, 12xdoublet, 1xtriplet, 21xquadruplet, 6xmultiplet	medium

LEUCINE	6	5	1	2xdoublet, 1xquadruplet, 3xmultiplier	medium
LYSINE	7	7	0	2xtriplet, 5xmultiplier	low
MALATE	3	3	0	3xquadruplet	low
MALONATE	1	0	1	1xsinglet	low
MALTOSE	28	28	0	4xdoublet, 3xtriplet, 17xquadruplet, 4xmultiplier	low
MANNITOL	4	4	0	1xdoublet, 2xquadruplet, 1xmultiplier	low
MELATONIN	10	9	1	4xsinglet, 1xtriplet, 3xdoublet, 2xquadruplet,	medium
METHANOL	1	1	0	singlet	low
METHIONINE	5	5	0	1xsinglet, 1xtriplet, 1xquadruplet, 2xmultiplier	low
METHYLAMINE	1	0	1	singlet	low
METHYLGUANIDINE	4	3	1	singlet, 3xmultiplier	low
METHYLMALONATE	2	2	0	1xdoublet, 1xquadruplet	low
METHYLSUCCINATE	4	3	1	1xdoublet, 2xquadruplet, 1xmultiplier	medium
N-ACETYL SEROTONIN	9	9	1	3xsinglet, 3xdoublet, 1xtriplet, 2xquadruplet	medium
N-METHYLHYDANTOIN	2	1	1	2xsinglet	medium
N-NITROSODIMETHYLAMINE	2	1	1	2xsinglet	medium
N-PHENYLACETYLGLYCINE	6	3	3	2xsinglet, 1xdoublet, 3xmultiplier	high
N,N-DIMETHYLFORMAMIDE	3	2	1	2xsinglet, 1xdoublet	medium
N,N-DIMETHYLGLYCINE	2	1	1	2xsinglet	medium
N6-ACETYLLYSINE	11	10	1	2xsinglet, 1xtriplet, 2xquadruplet, 6xmultiplier	medium
NÎ±-ACETYLLYSINE	12	12	0	1xsinglet, 1xdoublet, 10xmultiplier	low
O-ACETYLCARNITINE	7	7	0	2xsinglet, 1xdoublet, 3xquadruplet, 1xmultiplier	low
O-ACETYLCHOLINE	4	4	0	2xsinglet, 2xmultiplier,	low

O-PHOSPHOCHOLINE	3	2	1	1xsinglet, 2xmultiplet	low
P-CRESOL	3	2	1	1xsinglet, 2xmultiplet	medium
PANTOTHENATE	9	8	1	3xsinglet, 2xdoublet, 1xsinglet, 2xquadruplet, 1xmultiplet	medium
PHENYLACETATE	4	4	0	1xsinglet, 3xmultiplet	low
PHENYLALANINE	6	6	0	3xmultiplet, 3xquadruplet	low
PYRIDOXINE	4	3	1	4xsinglet	medium
PYRUVATE	1	0	1	singlet	low
QUINOLINATE	3	3	0	3xquadruplet	low
RIBOFLAVIN	11	10	1	4xsinglet, 1xtriplet, 3xquadruplet, 3xmultiplet	medium
SARCOSINE	2	1	1	2xsinglet	low
SERINE	3	3	0	3xquadruplet	low
SN-GLYCERO-3-PHOSPHOCHOLINE	8	7	1	1xsinglet, 2xquadruplet, 5xmultiplet	medium
SUCCINATE	1	1	0	singlet	low
SUCCINYLACETONE	4	3	1	2xsinglet, 2xtriplet	medium
SUCROSE	14	12	2	4xdoublet, 4xtriplet, 4xquadruplet, 2xmultiplet	high
TARTRATE	1	0	1	singlet	low
TAURINE	2	2	0	2xtriplet	low
THEOPHYLLINE	3	1	2	3xsinglet	medium
THREONATE	4	3	1	1xdoublet, 2xquadruplet, 1xmultiplet	medium
THREONINE	3	2	1	2xdoublet, 1xmultiplet	medium
TRANS-ACONITATE	2	1	1	2xsinglet	low
TRIGONELLINE	5	2	3	2xsinglet, 2xdoublet, 1xtriplet	high
TRIMETHYLAMINE	1	0	1	singlet	low
TRIMETHYLAMINE N-OXIDE	1	0	1	singlet	low
TRYPTOPHAN	9	6	3	2xsinglet, 2xdoublet, 2xtriplet, 3xquadruplet	high

TYROSINE	5	3	2	3xquadruplet, 2xmultiplet	high
UDP-GLUCOSE	15	14	1	3xdoublet, 4xtriplet, 3xquadruplet, 5xmultiplet	medium
URACIL	2	2	0	2xdoublet	low
UREA	1	0	1	singlet	low
VALINE	4	2	2	3xdoublet, 1xmultiplet	high
VANILLATE	4	3	1	1xsinglet, 2xdoublet, 1xquadruplet	medium
XANTHINE	1	0	1	singlet	low
XANTHOSINE	7	6	1	1xsinglet, 1xdoublet, 1xtriplet, 4xquadruplet	medium
π-METHYLHISTIDINE	6	5	1	3xsinglet, 3xdoublet	medium
τ-METHYLHISTIDINE	6	5	1	3xsinglet, 3xdoublet	medium

Appendix - Table 12: Confidence table of assigned urine metabolites detected with ^1H NMR spectroscopy. Metabolites were considered part of a peak if the Chenomx Profiler® software detected more than 10% contribution to a peak. For each metabolite the number of features is listed which can be a singlet, doublet, triplet, quadruplet or a multiplet. Column three and four indicate the number of overlapped and non-overlapped peaks. Confidence was determined using the number of features and overlaps. Low= one singlet, or several features, but all overlapping; medium= high number of distinct features or one feature not overlapped; high= several features not-overlapped or one feature not-overlapped if in a very distinct region of the ppm scale/very distinct feature.