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The vasculopathy of Juvenile Dermatomyositis

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Submitted for the degree of Doctor of Philosophy

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I, Charalampia Papadopoulou confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

Acknowledgments

First of all, I would like to thank from the bottom of my heart my primary supervisor, Dr Despina Eleftheriou, for being such a great mentor throughout this journey. Dr Eleftheriou thank you so much for all your support, your endless guidance and immense knowledge. We have been together through both difficult and good days, but I always felt that you were by my side and kept me going and helped me grow in every possible way. I am so grateful for all your patience as I definitely would not have made it without you. I would also like to thank Professor Paul Brogan for his insightful comments and encouragement, but also for the hard questions he asked that made me to widen my way of thinking and my perspectives. Special thanks to Dr Yiannis Ioannou who has been with me only at the beginning of this journey but had always ways to help and support me when things were getting difficult. I would not be able to find the right words to thank Ying Hong for her invaluable help and for teaching me that I should never be disappointed as there is always a solution to all problems. Thank you to Ebun, Annette, Marina, Dara, James for all their help and patience to my stupid questions. So many thanks to my partners in crime Athina and Elena for listening to me and joining me to my fresh air sections when I needed few minutes to relax. Very special thanks to Muthana for looking after me throughout these 4 years, being so stoic with my endless moaning and always knowing how to cheer me up. I would also like to thank my parents, my sister and her lovely family for always being there for me. Last, but definitely not least, my wonderful husband for all his love and understanding and for being there for me in all the ups and down of this journey.

Charalampia Papadopoulou, March 2019

Thesis abstract

Vasculopathy is considered central to the pathogenesis of Juvenile Dermatomyositis (JDM). One major hurdle to the study and detection of vasculopathy of JDM and to the monitoring of its progression over time has been the lack of non-invasive biomarkers. Therefore, defining disease activity trajectories relating to persistent endothelial injury in JDM currently remains challenging. This thesis addressed this unmet need by examining biomarkers of endothelial injury, subclinical inflammation and hypercoagulability in a large cohort of JDM patients. Circulating endothelial cells (CEC) were higher in patients with active JDM compared to patients with inactive disease and healthy controls. Total circulating microparticles (MP) counts were also significantly higher in JDM patients compared to healthy controls. These circulating MP were predominantly of platelet and endothelial origin. Enhanced plasma thrombin generation was demonstrated in active compared to inactive JDM and controls. When the inflammatory protein profile associated with endothelial activation and dysfunction in children with JDM was investigated, a number of cytokines/chemokines and adhesion molecules were shown to be elevated in patients with JDM. Levels of galectin-9 strongly correlated with other markers of disease activity. Lastly, increased arterial stiffness was also detected in children with JDM, suggestive of an increased risk of cardiovascular disease and premature atherosclerosis. In conclusion, this thesis demonstrated: 1. Increased endothelial injury in children with active JDM associated with high levels of CEC and circulating MP with propensity to drive thrombin generation and hence occlusive vasculopathy; and 2. Increased arterial stiffness in paediatric patients with JDM. These novel non-invasive biomarkers relating to the vasculopathy of JDM can now be used to track

endothelial injury relating to subclinical disease activity in JDM over time and may facilitate development of stratified treatment approaches to reduce long-term adverse outcomes for these children.

Impact statement

The observations I made during this PhD project suggest that children with active Juvenile Dermatomyositis (JDM) are characterized by ongoing endothelial injury and are on a chronic prothrombotic state that may contribute to the vascular complications associated with poorer disease outcomes. Although the overall prognosis of JDM has improved significantly over the recent years, the long-term outcome differs substantially from patient to patient. The lack of non-invasive biomarkers for the detection and monitoring of vascular injury in children with JDM makes prediction of these different disease trajectories challenging. The primary beneficiaries of this project will be patients with JDM. The major way in which patients with this disease will benefit from this study are summarised below:

i. Development of a panel of new biomarkers for use in clinical care across other UK centres able to predict disease severity and complications associated with vascular injury and thrombosis in JDM.
ii. Development of a scientific rationale for therapeutic stratification of individual patients and inform the use of novel targeted therapies such as Janus Kinase inhibitors that act directly downstream of interferon activation and may thus abrogate the interferon induced endothelial injury; and oral anti-thrombin agents to reduce

pathological thrombin generation. In fact, discussions have already begun between our group and with industry partners and we are liaising with national advisory bodies in order to advance this proposal for an interventional clinical trial in this rare disease. Therefore, findings of this thesis can directly inform this next translational phase of the overall project.

iii. Lastly, the cardiovascular morbidity relating to JDM is an under-researched area. My study provides evidence that children with active JDM have increased arterial stiffness. If evidence of premature atherosclerosis in JDM is confirmed, the use of new therapeutic approaches mentioned above, and also the institution of formal therapeutic lifestyle interventions may reduce the risk of accelerated cardiovascular morbidity before these patients progress into adulthood. These discoveries have the potential to significantly impact on the quality of life, health and well-being of these children as they progress to adulthood.

Academics throughout the world working in the field will benefit from new insight into the pathogenesis of JDM gained by studying the potential contribution of endothelial injury and thrombotic propensity to occlusive vasculopathy. Some of the data generated in this PhD has been publicised through open access peer review journals whilst the remainder is soon to be publicised hence contributing to significant knowledge enhancement and scientific advancement about the pathobiology of DM for academic beneficiaries in the field. Lastly, this PhD fellowship enabled my training as a skilled researcher and facilitated my academic career progress. I have developed expertise, knowledge and a deeper understanding of

investigative immunology and translational research. My project also contributes towards the health of the rheumatology academic discipline with publications and presentations.

Abbreviations

Abbreviation	Definition
APTT	Activated partial thromboplastin time
ACR	American College of Rheumatology
AECA	Anti-endothelial cell antibodies
ALT	Alanine aminotransferase
ANA	Antinuclear antibody
ANCA	Anti-neutrophil cytoplasmic antibodies
Ang	Angiopoietin
AnV	Annexin V
APC	Allophycocyanin
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUC	Area under the curve
BSA	Bovine serum albumin
BV	Brilliant violet

CAA	Coronary artery aneurysm
CARRA	Childhood Arthritis and Rheumatology Research Alliance
CEC	Circulating endothelial cells
CHAQ	Childhood Health Assessment Questionnaire
CI	Confidence interval
CIMT	Carotid-intima-media thickness
CK	Creatinine kinase
CMAS	Childhood Myositis Assessment Scale
CNS	Central nervous system
CRP	C-reactive protein
CTI	Corn trypsin inhibitor
CTLA-4	Cytotoxic T-lymphocyte associated antigen 4
CTP	Consensus Treatment Plans
CVD	Cardiovascular disease
CyC	Cyclophosphamide
DAH	Diffuse alveolar haemorrhage
DAS	Disease activity score
DC	Dendritic cells
DM	Dermatomyositis
DMARD	Disease-modifying anti-rheumatic drugs

DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMG	Electromyographic
EMP	Endothelial microparticles
ERL	End-row loop
ESR	Erythrocyte sedimentation rate
ETP	Endogenous thrombin potential
EULAR	European League Against Rheumatism
FCS	Fetal Calf Serum
FDP	Fibrin degradation products
FITC	Fluorescein isothiocyanate
FMD	Flow-mediated dilatation
FMO	Fluorescence Minus One
FSC-A	Forward scatter area
GOSH	Great Ormond Street Hospital NHS Foundation Trust
Hb	Haemoglobin
HCQ	Hydroxychloroquine
HLA	Human leukocyte antigen

HMCGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IIM	Idiopathic Inflammatory Myopathies
IL	Interleukin
ILD	Interstitial lung disease
IMACS	International Myositis Assessment & Clinical Studies Group
IP-10	Interferon gamma-induced protein 10
IQR	Interquartile range
ISG-15	IFN stimulated gene 15
IV	Intravenous
IVIG	Intravenous immunoglobulins
JAK	Janus Kinase
JDCBS	Juvenile Dermatomyositis Cohort and Biomarker Study
JDM	Juvenile Dermatomyositis
LDH	Lactate dehydrogenase
LR	Likelihood ratio
MAA	Myositis associated antibodies
MAC	Membrane attack complex
MAS	Macrophage activated syndrome

MCP	Monocyte chemoattractant protein
MDA5	Melanoma differentiation-associated gene 5
MEP	Methylprednisolone
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMF	Mycophenolate mofetil
MMT-8	Manual Muscle Testing -8
MP	Microparticle
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSA	Myositis specific antibodies
MSD	MesoScale Discovery
MTX	Methotrexate
MYOGEN	Myositis Genetics Consortium
NanoFACS	Nanoscale Fluorescence activated cell sorting
NMP	Neutrophil derived microparticles
NTA	Nanoparticle tracking analysis
NuRD	Nucleosome remodeling-histone deacetylase
NXP2	Nuclear matrix protein 2

PAI	Plasminogen activator inhibitor
ParGLOVAS	Parent's global assessment on a visual analogue score
PBAC	PBS containing 0.1% BSA and 0.6% sodium citrate
PBMC	Peripheral Blood Mononuclear cell
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	R-phycoerythrin
PEM	Pulmonary embolism
PerCP	Peridinin-chlorophyll-protein
PhyGLOVAS	Physician Global Assessment on a visual analogue score
PLT	Platelets
PM	Polymyositis
PMP	Platelet derived microparticles
PPP	Platelet poor plasma
PRES	Posterior reversible encephalopathy syndrome
PRINTO	Paediatric Rheumatology International Trials Organization
PS	Phosphatidylserine
pSTAT	Phosphorylated Signal Transducer Activator and Transcription
PT	Prothrombin time
PTPN22	Protein tyrosine phosphatase N22

PWV	Pulse wave velocity
qPCR	Quantitative reverse transcription polymerase chain reaction
RA	Rheumatoid arthritis
RCT	Randomised controlled trial
RNA	Ribonucleic acid
ROC	Receiver operator characteristic
RR	Reference range
RTX	Rituximab
SAA	Serum amyloid A
Scl	Scleroderma
SE	Standard Error
SHARE	Single Hub and Access point for paediatric Rheumatology in Europe
SLE	Systemic lupus erythematosus
snRNP	Ribonucleoprotein
SRP	Signal recognition particle
SSc	Systemic sclerosis
SSC-A	Side scatter area
STAT	Signal transducer and activator of transcription
TF	Tissue factor
TGA	Thrombin generation assay

Th17	T helper type 17
TIF	Transcription intermediary factor
TLR	Toll Like Receptor
TM	Thrombomodulin
TNF	Tumour Necrosis Factor
TNFRII	Tumour Necrosis Factor Receptor II
TNF-α	Tumour Necrosis Factor-α
t-PA	Tissue-type plasminogen activator
TYK	Tyrosine kinase
UCL	University College London
u-PA	Urokinase-type plasminogen activator
VAS	Visual Analogue Score
VCAM-1	Vascular cell adhesion molecule-1
VIP	Vasculitis and Inflammation Panel
VTE	Venous thrombotic events
WCC	White cell count

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1 Introduction

1.1 Definitions

Idiopathic Inflammatory Myopathies (IIM) include a number of highly heterogeneous diseases characterized by systemic inflammation mainly affecting the muscles and the skin, but on occasions involvement of other organs as well (Wedderburn and Rider, 2009). Among IIM, Juvenile dermatomyositis (JDM) is the commonest subgroup, accounting for approximately 85% of the cases (Ramanan and Feldman, 2002; McCann *et al.*, 2006); and less than 5% of paediatric IIM cases belong to the juvenile polymyositis (JPM) category (McCann *et al.*, 2006; Ravelli *et al.*, 2010).

The Peter and Bohan diagnostic criteria for JDM were first described in 1975 (Bohan and Peter, 1975) and include pathognomonic cutaneous changes, symmetrical weakness of the proximal muscles, increased muscle enzymes, characteristic muscle biopsy findings including necrosis and inflammation, and typical electromyographic (EMG) changes. For a possible diagnosis of JDM, the presence of pathognomonic cutaneous changes is required in combination with two other of the above-mentioned criteria; for a definite diagnosis the presence of characteristic skin changes along with three of the other four criteria is required. In an international survey published in 2006, typical clinical features (proximal muscle weakness and pathognomonic skin rashes) and laboratory findings (elevated muscle enzymes) were widely used to establish a diagnosis of JDM (Brown *et al.*, 2006). Approximately 60% of paediatric rheumatologists considered magnetic resonance imaging

(MRI) as an important diagnostic tool for the detection of inflammatory muscle changes (Brown *et al.*, 2006). Muscle biopsy was used by only 61.3% of responders, as it is an invasive procedure. Similarly, EMG was only used by 55.5% of responders to establish a diagnosis of JDM as it is also a relatively invasive procedure (Brown *et al.*, 2006). A muscle biopsy, may however have a role in facilitating diagnosis in cases of polymyositis (PM) (Oddis *et al.*, 2005). In 2007, Wedderburn *et al*. proposed a scoring tool for measuring the severity of histological findings in JDM in a standardised way which has since been widely used (Wedderburn *et al.*, 2007c). This score consists of 4 domains: inflammatory, vascular, muscle fibre, and connective tissue. In addition to the 4 domains, an overall score of severity derived by marking a Visual Analogue Score (histopathologists' VAS) (1.0–10.0 cm) is also included (**Appendix 1**).

Taking into account the decreasing use of muscle biopsy and EMG in routine clinical practice, it has become evident that a number of children would fail to meet the current (but now rather outdated) diagnostic criteria often cited as necessary for the inclusion in research and other collaborative studies. For that reason, in 2017 the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) endorsed and semi-validated revised classification criteria for adult and juvenile IIM (Lundberg *et al.*, 2017). These criteria include four muscle related variables, three skin related variables and other laboratory variables and clinical manifestations. As muscle biopsy is not always performed in children, a different scoring system has been proposed for when a muscle biopsy is available and when it is not (**Table 1.1**). In the presence of heliotrope rash, Gottron's papules and/or Gottron's

sign, patients with myositis are classified as having JDM and, respectively, in the absence of skin rashes patients are classified as having JPM. These new criteria, are however, classification criteria and not diagnostic criteria, therefore should not be misused as the latter.

Variable	**Score Points**	
	Without muscle biopsy	**With muscle biopsy**
Muscle weakness		
Objective symmetric weakness, usually progressive, of the proximal upper extremities	0.7	0.7
Objective symmetric weakness, usually progressive, of the proximal lower extremities	0.8	0.5
Neck flexors are relatively weaker than neck extensors	1.9	1.6
In the legs proximal muscles are relatively weaker than distal muscles	0.9	1.2
Skin manifestations		
Heliotrope rash	3.1	3.2
Gottron´s papules	2.1	2.7
Gottron's sign	3.3	3.7
Other clinical manifestations		
Dysphagia or oesophageal dysmotility	0.7	0.6
Laboratory measurements		
Anti-Jo-1 (anti-histidyl-tRNA synthetase) autoantibody present	3.9	3.8
Elevated serum levels of	1.3	1.4

Variable	Score Points	
	Without muscle biopsy	**With muscle biopsy**
creatine kinase (CK) *or* lactate dehydrogenase (LDH) *or* aspartate aminotransferase (AST) *or* alanine aminotransferase (ALT)		
Muscle biopsy features- presence of:		
Endomysial infiltration of mononuclear cells surrounding, but not invading, myofibres		1.7
Perimysial and/or perivascular infiltration of mononuclear cells		1.2
Perifascicular atrophy		1.9
Rimmed vacuoles		3.1

Table 1.1. The European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) classification criteria for juvenile idiopathic inflammatory myopathies (IIM). *Adapted from Lundberg et al. (Lundberg et al., 2017). A patient is classified as having possible IIM if the total score is 5.3-5.4 if no muscle biopsy is available and 6.5-6.6 if a muscle biopsy is obtained. Patients are classified as having probable IIM if the total score is ≥5.5 to <7.5 in cases where no muscle biopsy is available and ≥6.7 to <8.7 if a muscle biopsy is available. Lastly, patients are classified as having definite IIM if the total score is ≥7.5 in cases where no muscle biopsy is available and ≥8.7 if a muscle biopsy is available. Patients who meet the criteria for IIM are then subclassified as having Juvenile dermatomyositis in the presence of heliotrope rash or gottron's papules or gottron's sign.*

1.2 Comparison with adult Dermatomyositis

IIM in adults is much more common than in children, with different cohorts reporting a 5-10 times higher incidence (Tansley and Wedderburn, 2015; Pachman and Khojah, 2018). Although JDM accounts for approximately 85% of the childhood IIM cases, dermatomyositis (DM) is seen in about 14%-28% of the adult cases (Pachman and Khojah, 2018). Regarding the clinical presentation, adult and childhood myositis share some common clinical features such as characteristic skin rashes, and inflammatory muscle changes. Adult onset DM and JDM are also highly diverse diseases with distinct additional clinical manifestations and distinct outcomes. There are however some features that are clearly unique to either adult or juvenile onset myositis (Tansley *et al.*, 2013). Vasculopathic features, development of calcinotic lesions, and ulcerative skin disease is seen at a much higher frequency in children than in adults with myositis. Adult cases are more commonly associated with malignancy and interstitial lung disease (Tansley and Wedderburn, 2015). These differences were highlighted in a recent large cohort study using data from the EuroMyositis registry, in which both adult and childhood onset cases of IIM were included (Lilleker *et al.*, 2018). Despite these differences in clinical presentation, much of the current knowledge of the pathophysiology of paediatric IIM derives from data from adult DM studies, due to the rarity of the juvenile onset disease.

1.3 Epidemiology

JDM is a rare autoimmune disease with a reported annual incidence ranging between 2 to 4 cases per one million children per year (Oddis *et al.*, 1990; Symmons *et al.*, 1995; Mendez *et al.*, 2003). The exact prevalence of JDM has not yet been reported. Females are more commonly affected than males, with reported female: male ratios ranging between 2:1 and 5:1 in different cohorts (Symmons *et al.*, 1995; Mendez *et al.*, 2003; Pachman *et al.*, 2006a). The reported median age of disease onset is seven years old (Symmons *et al.*, 1995; Pachman *et al.*, 1998), 6.7 years for boys, and 7.3 years for girls (Mendez *et al.*, 2003). Although JDM occurs worldwide, some studies suggest different incidence rates among different racial groups. Some of these studies have suggested that children of African, or Caucasian origin are more frequently affected (Mendez *et al.*, 2003).

1.4 Clinical manifestations of JDM

1.4.1 Systemic symptoms

Constitutional symptoms are frequent in JDM. These include fever, fatigue, irritability, anorexia, weight loss, and poor growth and may be present at either initial presentation of disease onset, or develop during the JDM disease course.

1.4.2 Muscle disease (muscle weakness, dysphagia and dysphonia)

Symmetric weakness of the proximal muscles is a central feature of JDM present in almost 95% of paediatric cases at the time of diagnosis (Feldman *et al.*, 2008). This may range from mild muscle weakness indicated by difficulty getting up from the floor, or difficulty turning

over in bed due to more severe symptoms, and even profound weakness with difficulty swallowing and breathing. The pelvic, shoulder and anterior flexor muscles of the neck and upper and lower body are mainly affected, but theoretically, any muscle can be affected (Rosa Neto and Goldenstein-Schainberg, 2010). Inflamed muscles can be painful and rigid, and the overlying subcutaneous tissue can be oedematous and indurated. Neck flexor muscle weakness is typical, and children are characteristically unable to lift their head up when lying flat on a bed. Gower's sign (difficulty in getting off the floor to achieve an upright position requiring the use of the hands) is also frequently present, suggestive of proximal lower limb muscle weakness. Although muscle weakness can be profound, deep tendon reflexes are usually preserved. Absence of muscle involvement (amyopathic JDM) is seen in <5% of children (Caproni *et al.*, 2002).

Involvement of the oropharyngeal, laryngeal and oesophageal musculature can lead to dysphagia for liquids and solids (McCann *et al.*, 2007). Such symptoms may be present in up to 29–44% children (Ramanan and Feldman, 2002; McCann *et al.*, 2006). Dysphagia is not always clinically present, and silent aspiration may occur. The recognition of an unsafe swallow is important to avoid aspiration leading to secondary pulmonary changes and damage. This can be prevented by nasogastric tube feeding. Nasal voice and dysphonia can be the result of palatal weakness. Oropharyngeal involvement has been associated with poorer outcomes (Taieb *et al.*, 1985; McCann *et al.*, 2007).

1.4.3 Arthritis

Arthritis is a common manifestation in patients with JDM, occurring in 23–64% of cases (Pachman *et al.*, 1998; McCann *et al.*, 2006). A recent study suggested that oligoarticular (<5 joints) joint involvement was present at diagnosis in 67% of patients, with polyarticular course in 33% of JDM cases (Tse *et al.*, 2001). Arthritis may occur early in the disease onset. It is usually non-erosive and responds well to steroid treatment. The main joints involved are the knees, wrists, elbows, and fingers. Affected joints can be painful. Flexion contractures particularly of large joints are common and usually develop early during the disease course. This may be the result of myofascial inflammation and may not necessarily be related to the presence of arthritis (Cassidy *et al.*, 2010).

1.4.4 Cutaneous manifestations

The JDM skin manifestations are numerous and varied (Lowry and Pilkington, 2009). Cutaneous manifestations may present months or even several years before muscle weakness, and may be the most difficult to treat feature of the JDM (Dugan *et al.*, 2009b). The most typical skin changes associated with JDM are discussed below in more detail.

1.4.4.1 Heliotrope rash

The heliotrope rash is violaceous, or reddish-purple in colour rash often involving the upper eye lids **(Figure 1.1)**. It is one of the most common diagnostic rashes seen in JDM. It is often associated with periorbital oedema, and accompanied by a malar rash (affecting both cheeks, and extending across the nasal bridge), similar to the malar rash seen in lupus, but usually not

sparing the nasolabial folds, and usually less well-demarcated than lupus (**Figure 1.2**). The chin and forehead can be involved; rashes can be photosensitive, with a reported prevalence of up to 86.7% of JDM cases (Shah *et al.*, 2013a). Heliotrope rash with Gottron's papules is pathognomonic of JDM at presentation, especially if periungual erythema and capillary loop abnormalities are present (Dugan *et al.*, 2009a).

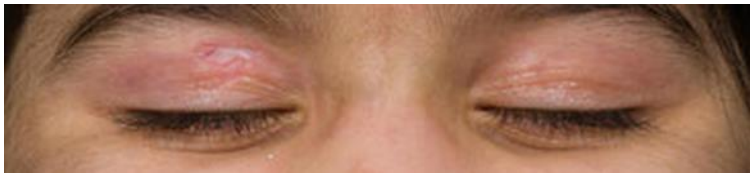

Figure 1.1. Heliotrope rash in a 5.5-year-old child with Juvenile dermatomyositis (JDM).

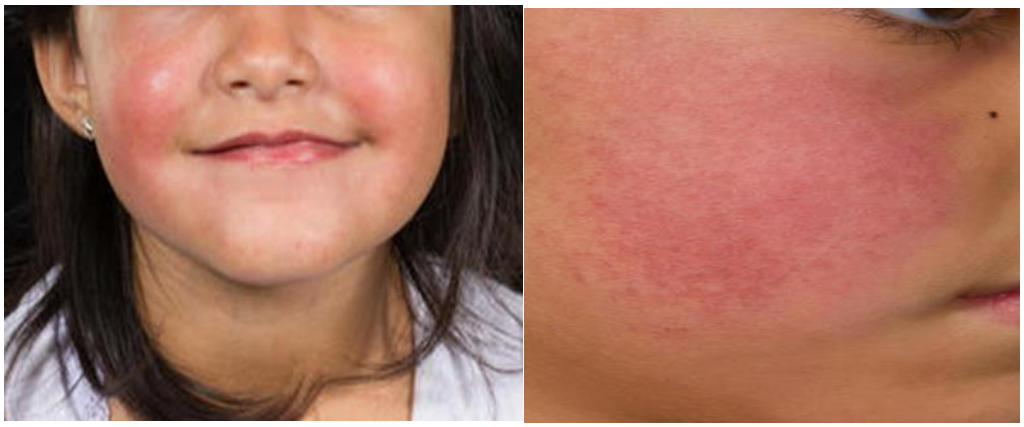

Figure 1.2. Malar rash in a 5.5-year-old child with Juvenile dermatomyositis (JDM).

1.4.4.2 Gottron's sign and papules

Gottron's sign is a flat red rash and gottron's papules are raised erythematous, shiny, scaly lesions on extensor surfaces especially in sun-exposed areas such as the extensor surfaces of the knees (**Figure 1.3**), the small joints of the hands (metacarpophalangeal and interphalangeal joints) (**Figure 1.4**), and the elbows, as well as the medial malleoli of the ankles. As Gottron's papules progress, the central area can become slightly depressed, and atrophic with a white appearance (Iaccarino *et al.*, 2014). No other inflammatory dermatological disease affects the knuckles in a similar fashion (Baykal and Polat Ekinci, 2015). As Gottron's papules are scaly lesions, they can be misdiagnosed as psoriasis. Ulceration and atrophic changes may be indicative of secondary damage within the papules (Dugan *et al.*, 2009a).

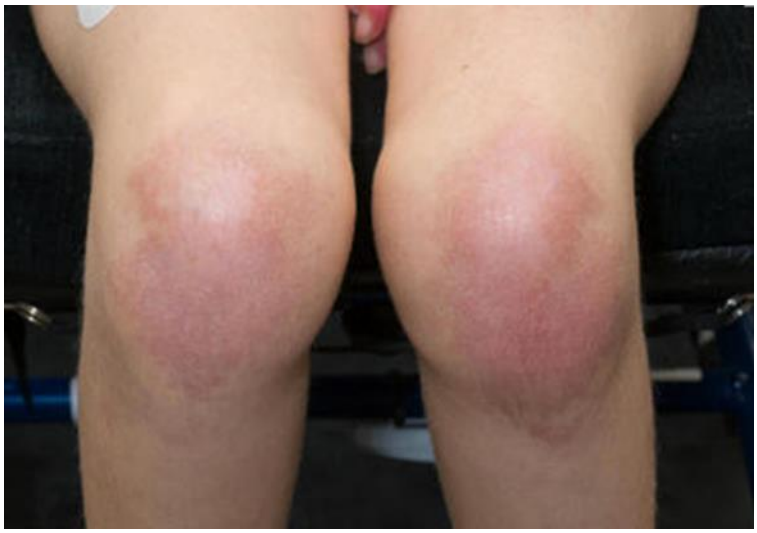

Figure 1.3. Gottron's papules over the extensor surface of the knees in a 5.5-year-old child with Juvenile dermatomyositis (JDM).

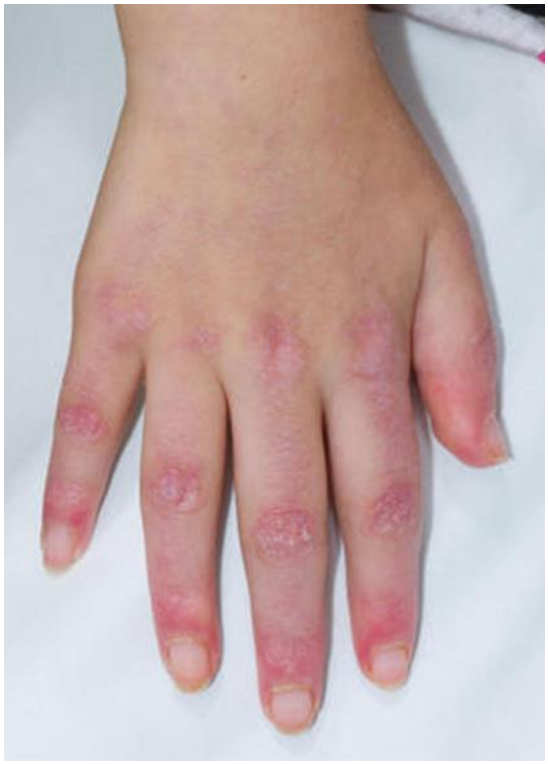

Figure 1.4. Gottron's papules over the metacarpophalangeal and interphalangeal joints in a 5.5-year-old child with Juvenile dermatomyositis (JDM).

1.4.4.3 Other types of skin rashes

There is a wide range of other skin rashes seen in children with JDM. The so-called V-neck sign is a violaceous erythema sometimes seen in the V area of the neck and the upper chest, while the symmetrical erythema located over the back and shoulders is called the shawl sign. Linear extensor erythema on the fingers is considered a sign of severe disease; scalp dermatitis and generalized pruritic rash have been described in several cohorts (Dugan *et al.*, 2009a; Cassidy *et al.*, 2010; Iaccarino *et al.*, 2014).

1.4.4.3.1 Nailfold capillary changes

The changes in the nailfold capillaries seen in JDM include capillary enlargement, occlusion, tortuous and arborized capillary loop clusters, disorganisation of the normal capillary distribution, micro-haemorrhages, and drop-out of normal capillary loops leading to decreased vessel number, and development of avascular areas (Spencer-Green *et al.*, 1982). These changes may be present in 80-91% of children at the time of diagnosis. Nailfold capillary changes are dynamic and change in appearance over the course of the disease and with treatment (Schmeling *et al.*, 2011; Manfredi *et al.*, 2015). Nailfold capillaroscopy is a non-invasive, inexpensive and simple technique that provides quantitative information about the loss of capillary end-row loop (ERL), changes in the vascularity of the studied areas, and the presence of arboreal loops (Dolezalova *et al.*, 2003; Christen-Zaech *et al.*, 2008). Capillaroscopy can also easily be performed at the bedside with the use of a light, a 10 x magnifying glass (dermatoscope, ophthalmoscope) and a water-soluble gel (Feldman *et al.*, 2008) placed on the nailfold bed to increase resolution. The skin around the nail beds can also be erythematous (peri-ungual erythema), and painful; cuticular overgrowth can be present (**Figure 1.5**). The peripheral micro-vasculopathic changes observed with nailfold capillaroscopy or video capillaroscopy correlate with a prolonged disease course, overall disease activity, skin disease activity, and poor response to treatment (Smith *et al.*, 2004; Christen-Zaech *et al.*, 2008; Gitiaux *et al.*, 2016). Nailfold capillary changes reflect the degree of systemic microangiopathy that characterises JDM (Schmeling *et al.*, 2011), and have been demonstrated to correlate with the development of other organ involvement, such as interstitial lung disease (Smith *et al.*, 2013) and gastrointestinal involvement (Gitiaux *et al.*,

2016). Similar microvascular changes have also been described in the gingiva of children with JDM as part of the vasculopathy (Rider and Atkinson, 2009).

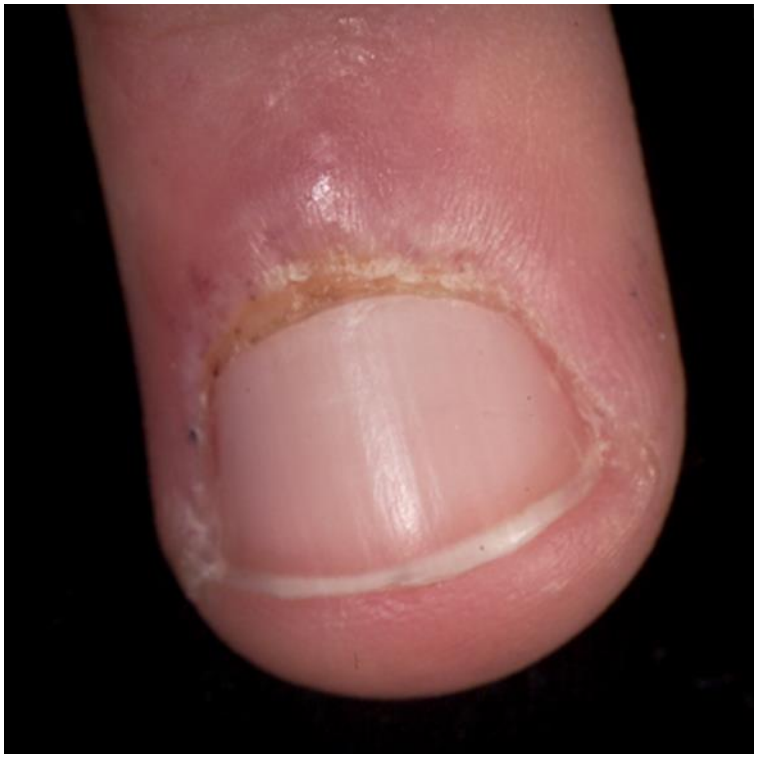

Figure 1.5. Periungual erythema in a 5.5-year-old child with Juvenile dermatomyositis (JDM).

1.4.4.4 Skin ulceration

Skin ulceration can complicate JDM and represents a potentially life-threatening clinical feature. The development of skin ulcers is indicative of severe vasculopathy of the skin caused by significant obstruction in blood flow, hypoxia and subsequent ischaemia of the affected tissues. This may indicate vasculopathy in other organs (e.g. intestinal ischaemia and

perforation, pulmonary fibrosis and interstitial lung disease) (Pachman and Cooke, 1980; Mamyrova *et al.*, 2007). The presence of ulcerative skin disease is associated with a more severe disease course with a poorer long-term prognosis (Bowyer *et al.*, 1983). It is present in approximately 23-30% of patients (Feldman *et al.*, 2008) with JDM and is more commonly seen at the corner of the eyes **(Figure 1.6)**, over elbows or other pressure points (Crowe *et al.*, 1982). Theoretically any part of the body can be affected, however.

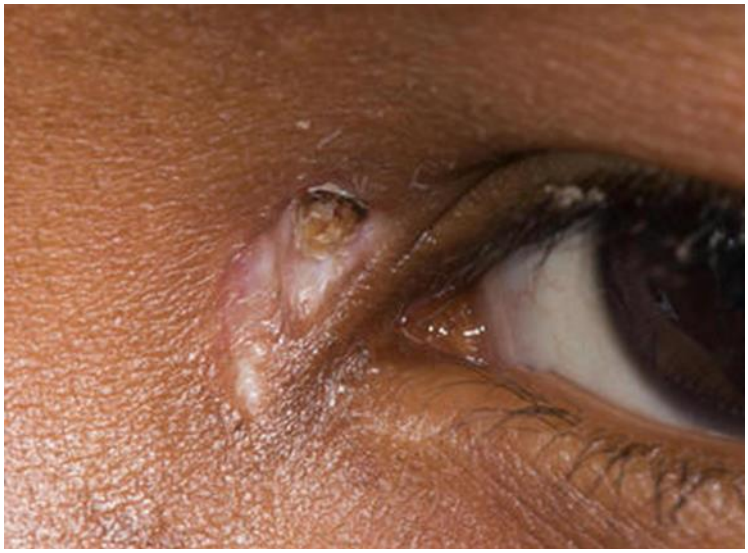

Figure 1.6. Healing vasculitic ulcer at the corner of the left eye in a 6.5-year-old boy with Juvenile dermatomyositis (JDM).

1.4.4.5 Calcinosis

Calcinosis is seen in 20-40% of JDM patients, although rarely seen in adults with DM (Pachman *et al.*, 2006b). The deposits are firm, flesh or white -coloured nodules that mainly occur over bony prominences, and have a high mineral content of calcium hydroxyapatite, osteonectin, osteopontin, and bone sialoprotein (Pachman *et al.*, 2006b). Sites that are mostly affected

include the knees, elbows, and upper and lower limbs, but calcinosis can occur in any part of the body (**Figure 1.7, 1.8**). Calcinosis usually occurs within 3 years of disease diagnosis, but it may be seen up to twenty years later (Rider, 2003). Dystrophic calcinotic lesions can result in skin ulceration. Fixed flexion deformities of the joints can occur when calcinotic lesions overlie the joint and may also extend outside the joint margins (Huber *et al.*, 2000). Neuropathic pain due to nerve entrapment may also develop, and there may be localised inflammation causing significant pain and swelling that can be misdiagnosed as cellulitis (Moore *et al.*, 1992; Feldman *et al.*, 2008). The pathogenesis of calcinosis is very poorly understood (Pachman *et al.*, 2000). Whether the development of calcinosis reflects active disease, or is the "damage" end result of chronic uncontrolled disease is debatable (Orandi *et al.*, 2017).

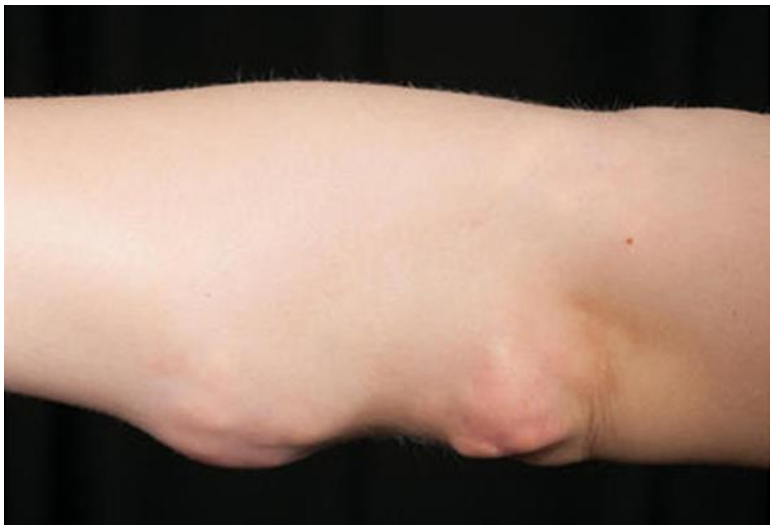

Figure 1.7. Calcinosis over the elbow in a 7-year-old child with Juvenile dermatomyositis (JDM).

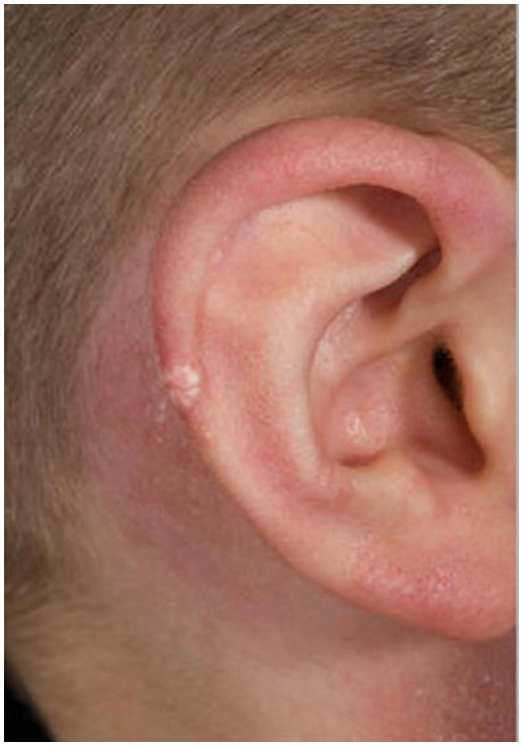

Figure 1.8. Small calcinotic lesion on the ear lobe in an 8-year-old child with Juvenile dermatomyositis.

1.4.5 Oedema

Subcutaneous oedema can be present in approximately 32% of JDM patients as reported in a UK cohort (Lovell *et al.*, 1999). The development of oedema may indicate a more severe disease course with resistance to treatment, and is usually observed at the time of initial diagnosis, or may develop during a severe disease flare (Mitchell *et al.*, 2001). Vascular endothelial injury and damage results in generalised capillary leak that leads to the development of subcutaneous oedema (Mitchell *et al.*, 2001). The presence of oedema at presentation may be misdiagnosed as nephrotic syndrome, but the absence of proteinuria or significant hypalbuminaemia, in combination with the characteristic skin rashes usually facilitates the diagnosis.

1.4.6 Cardiac involvement

Several cardiac abnormalities have been reported in children with JDM. Subclinical left ventricular diastolic dysfunction is observed in about 25% of JDM patients; and several electrocardiographic (ECG) abnormalities are reported with a high incidence in recent case control studies (Na *et al.*, 2009; Schwartz *et al.*, 2014). Rosenbohm et al. found that about 62% of the patients with inflammatory myositis had late gadolidium enhancement in cardiac MRI scanning indicating subclinical myocardial inflammation (Rosenbohm *et al.*, 2015). Pericarditis has also been reported (Sallum *et al.*, 2008). JDM patients have also been found to have reduced heart rate variability which may relate to reduced cardiac vagal control (Barth *et al.*, 2016). Micro-vasculopathy and corticosteroid treatment have been considered to be responsible for the hypertension observed in 25% to 50% of JDM patients (Rider *et al.*, 2009; Gitiaux *et al.*, 2016). Persistence of cardiac dysfunction has also been associated with persistence of skin disease (Christen-Zaech *et al.*, 2008) suggesting perhaps that both skin and cardiac disease are the result of the same vasculopathic process. The long-term effect of this subclinical cardiac involvement and its possible contribution towards an increased cardiovascular morbidity later in adulthood is largely unknown. In the only study of adults with juvenile onset DM, subclinical cardiovascular disease and increased carotid-intima-media thickness was demonstrated in the majority of patients studied. These changes are suggestive of early onset atherosclerosis despite absence of other traditional cardiovascular risk factors (Eimer *et al.*, 2011). Further studies are needed to further evaluate the cardiovascular morbidity and long-term cardiac sequelae of JDM.

1.4.7 Pulmonary manifestations

Although not as common as in adults with IIM, pulmonary involvement can be a life-threatening complication of JDM. Development of interstitial lung disease (ILD) and fibrosis can complicate paediatric JDM cases (Kobayashi *et al.*, 2003; Mamyrova *et al.*, 2013). These features might relate to the presence of specific myositis specific antibodies (MSA), particularly serum anti-melanoma differentiation-associated gene 5 (MDA5) antibodies and anti-synthetase antibodies (Tansley *et al.*, 2014a). Typically, ILD is asymptomatic during the early stages, with increasing cough and progressive dyspnoea indicating disease progression. Of note, although ILD is rare, abnormal pulmonary function tests are recorded in more than half of JDM cases (Pachman, 1995; Trapani *et al.*, 2001). The exact pathogenetic mechanisms of pulmonary involvement are not fully understood. The same model of microvascular injury observed in nailfold capillaroscopy is thought to cause endothelial cell damage and alveolar epithelial injury (Castelino and Varga, 2010), leading to the release of numerous cytokines and growth factors which play a key role in the development of lung disease. Other rare but potentially fatal manifestations of JDM include diffuse alveolar haemorrhage (DAH) and pneumomediastinum (Kono *et al.*, 2000; Carneiro da Silval *et al.*, 2009; Dogra *et al.*, 2012), which are again considered the end result of pulmonary vasculitis, fibrosis, and infarction (Lim *et al.*, 2017).

1.4.8 Gastrointestinal vasculopathy

Gastrointestinal tract involvement occurs in 5%-37% of JDM cases. This includes dysphagia, bowel dysmotility, vasculitis with associated malabsorption, and other more severe features

of gastro-intestinal vasculopathy that can be life-threatening. The main presenting symptoms include abdominal pain and rectal bleeding which can be subsequently complicated by intestinal ischaemia, pneumatosis intestinalis, and finally gut perforation (Downey *et al.*, 1988). The underlying pathophysiology is not fully understood, but it is considered to be complex and multifactorial as indicated by the histopathological evidence of acute inflammatory vasculitis and chronic gastrointestinal occlusive arteriopathy (Mamyrova *et al.*, 2007). Persistent, severe abdominal pain is a worrying sign and further extensive radiographic imaging and investigations should be performed the soonest possible. The main radiographic finding is thickening of the bowel folds as demonstrated by barium enema and follow-through studies (Laskin *et al.*, 1999).

1.4.9 Other organ involvement

Central nervous system involvement has been rarely reported in children with JDM (Ramanan *et al.*, 2001) . CNS involvement may manifest with hallucinations and seizures (Elst *et al.*, 2003) and active retinal vasculitis may co-exist (Ramanan *et al.*, 2001; Elst *et al.*, 2003). However, a single centre retrospective review of 82 patients demonstrated that retinopathy was rare, and concluded that routine assessment was not warranted for patients without visual symptoms (Akikusa *et al.*, 2005).

1.4.10 Lipodystrophy

Lipodystrophy usually occurs several years after disease onset with a reported prevalence of 20-25% of JDM patients (Huang, 1996; Quecedo *et al.*, 1996; Huemer *et al.*, 2001). It may be

present only in a limited area of the body, but can also be more generalised affecting larger surfaces. In the affected areas, there is loss of the subcutaneous fat which usually progresses slowly and symmetrically affecting mainly the face and the upper half of the body (Bingham *et al.*, 2008) (**Figure 1.9**). JDM patients positive for the anti- transcription intermediary factor (TIF)1g antibody have been found to be at higher risk of developing lipodystrophy (Bingham *et al.*, 2008). Lipodystrophy can also be associated with other metabolic abnormalities such as dyslipidaemia, hirsutism, and clitoromegaly and irregular menstrual cycle in girls (Pope *et al.*, 2006).

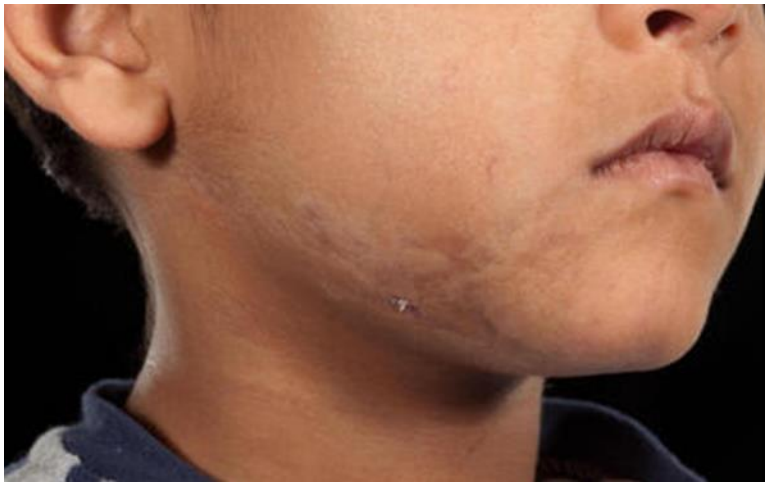

Figure 1.9. Lipodystrophy over the right side of the face in an 8-year-old with Juvenile dermatomyositis.

1.4.11 Macrophage activation syndrome (MAS) in JDM

MAS is well described in rheumatic diseases and the reported mortality rate reaches 10% (Lin *et al.*, 2012). It is more often seen in children with Systemic Onset Juvenile Idiopathic Arthritis,

and juvenile systemic lupus erythematosus (SLE) (Ravelli *et al.*, 2012). It has been reported in JDM (Lilleby *et al.*, 2014; Wakiguchi *et al.*, 2015), but is not as common. MAS is characterised by prolonged fever, enlarged lymph nodes, liver and spleen enlargement, reduced cell counts (anaemia, leukopenia, thrombocytopenia), increased C-reactive protein, falling trend in the levels of erythrocyte sedimentation rate, low fibrinogen, abnormal liver enzymes, elevated triglycerides and extreme levels of serum ferritin. It is often related with multi-organ dysfunction, and death if not treated. It is very important that clinicians are aware of the possibility of MAS as early recognition and aggressive treatment is key to a successful outcome.

1.4.12 Metabolic abnormalities (osteoporosis, dyslipidaemia, insulin resistance)

Osteopenia or osteoporosis are present in the majority of patients with IIM, even before starting treatment with corticosteroids. Several factors may be contributing to the development of osteopenia, including long-term use of high dose corticosteroids, persistent activity of the disease, reduced calcium intake, reduced exposure to sunlight, and prolonged immobility (Perez *et al.*, 1994; Falcini *et al.*, 2000). Early diagnosis and treatment are important (Castro *et al.*, 2005). JDM has also been associated with other metabolic disorders. Hyper-triglyceridaemia and low levels of high-density lipoproteins were observed in a recent study (Kozu *et al.*, 2013). Moreover, insulin resistance, increased body mass index and hypertension can be present in up to 50% of patients, which in combination with the sedentary lifestyle and chronic systemic inflammation act as risk factors for premature cardiovascular morbidity later in adulthood (Cassidy *et al.*, 2010).

1.5 Myositis specific antibodies

Recently, specific autoantibodies have been identified which can further define childhood IIM cases into more homogeneous groups with specific phenotypes (Love *et al.*, 1991; Mimori *et al.*, 2007; Wedderburn *et al.*, 2007a). These autoantibodies include the myositis specific antibodies (MSA) and myositis associated antibodies (MAA), which are present in about 60% of cases (Tansley and Wedderburn, 2015). MSA are considered specific for inflammatory myopathies (Mammen *et al.*, 2015; Tansley *et al.*, 2017), while MAA are also seen in other connective tissue diseases and overlap syndromes (Tansley *et al.*, 2017). Each autoantibody has (somewhat preliminarily) been associated with a specific phenotype which may differ based on the age of disease onset (Tansley and Wedderburn, 2015) (**Table 1.2**). The coexistence of more than one MSA in the same patient is very rare (Tansley *et al.*, 2017). The most sensitive method for identification of MSA is protein immunoprecipitation (Tansley *et al.*, 2017) but highly specialised enzyme-linked immunosorbent assay (ELISA) (Tansley *et al.*, 2017) and line blot assays (Ghirardello *et al.*, 2010) have also been used. Specific MSA have been associated with specific disease risk alleles (Wedderburn and Rider, 2009; Rider and Nistala, 2016). Each MSA targets a specific intracellular antigen with significant role in gene regulation and protein transcription and translation (McHugh and Tansley, 2018) (**Table 1.2**).

Antibodies directed against the transcription intermediary factor 1 g (TIF1g)/p155/140 are the commonest MSA in children with IIM, and are present in 23–29% of patients with JDM. The presence of anti-TIF1g antibodies is associated with severe cutaneous disease, ulcerative skin disease, lipodystrophy, and a chronic disease course (Gunawardena *et al.*, 2008). Anti-

nuclear matrix protein 2 (NXP2)/p140 antibodies are mainly associated with the development of calcinosis, more severe muscle weakness, gastrointestinal involvement and dysphagia in paediatric cases of JDM (Gunawardena *et al.*, 2009; Tansley *et al.*, 2014b) and are present in 13%-23% of the cases. Anti-melanoma differentiation-associated gene 5 (MDA5) antibodies are the third most common category of MSA and are associated with mild muscle disease, the presence of arthritis mainly of the small joints, ulcerative skin disease and increased risk of ILD (Tansley *et al.*, 2014a). Anti-MDA5 antibodies are seen in about 6% of childhood IIM with the exception of the Japanese population where they represent the commonest MSA subgroup present in 54% of JDM cases (Kobayashi *et al.*, 2015). These antibodies are also associated with high levels of interleukin (IL) -6, -18 and hyperferritinaemia (Kobayashi *et al.*, 2015). The exact mechanism of hyperferritinaemia in those patients remains unclear. Anti-Mi2 antibodies are described in approximately 4% of JDM cases in different cohorts (Rider and Nistala, 2016; Tansley *et al.*, 2017) and target the nucleosome remodeling-histone deacetylase (NuRD) subunit. These antibodies are associated with a typical JDM phenotype (presence of characteristic JDM rash) and usually a good long-term prognosis (Deakin *et al.*, 2016; Pachman and Khojah, 2018). Antibodies against the t-ribonucleic acid (RNA)-synthetase antigens are present in less than 5% of paediatric IMM, seen in older children and relate to risk of ILD, mechanic's hands (thickened skin of tips and margins of the fingers), muscle and joint inflammation and Raynaud's phenomenon (Tansley *et al.*, 2017). Two antibodies have been associated with necrotizing myopathy, with significantly elevated muscle enzymes and evidence of necrosis in muscle biopsies: the anti-signal recognition particle (SRP); and anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) antibodies. These may be present

in 2-5% and 1 % of patients, respectively (Rouster-Stevens and Pachman, 2008; Allenbach *et al.*, 2014; Kishi *et al.*, 2017). Finally, a number of MAA are associated with overlap syndromes, including antibodies against U1 ribonucleoprotein (snRNP), Polymyositis/Scleroderma (PM/Scl) and Ro-52 antibodies (Rider *et al.*, 2013)(**Table 1.2**).

Although not representing MSA, it is of great interest that anti-endothelial cell antibodies (AECA) have been recently identified in the plasma of JDM cases (Karasawa *et al.*, 2018). Seventy-six % of JDM patients were found to be positive for AECA compared to 30% in healthy controls (Yu *et al.*, 2016). When possible antigens of AECA were investigated, 22 possible target antigens were suggested in the plasma of JDM patients, the majority of which were proteins associated with antigen processing and protein trafficking (Karasawa *et al.*, 2018). AECA are associated with the presence of vasculitis and vascular thrombosis in lupus patients (Cieslik *et al.*, 2008).

It is well recognised that the levels of MSA fluctuate during disease course and can become undetectable with treatment and control of disease activity. Moreover, there is no evidence whether specific antibodies pre-date the development of specific disease features. If so, they could be useful as prognostic biomarkers, support early aggressive treatment and guide further therapeutic management (Tansley and Wedderburn, 2015), but at the moment this is aspirational, and no biomarker exists that can reliably do this. In support of this concept, however, Deakin et al. demonstrated that MSA, in combination with muscle biopsy severity score, might be predictive of long-term treatment status and prognosis in children with JDM

(Deakin *et al.*, 2016), although the strength of that association was somewhat weak: odds ratio 1.48 (95% confidence interval [95% CI] 1.12–1.96; *P* = 0.0058) for the histopathologist's visual analog scale score and odds ratio 1.10 (95% CI 1.01–1.21; *P* = 0.038) for the total biopsy score, respectively. Moreover, there were several limitations to this study: this was a retrospective study, without clinician blinding to muscle biopsy results and (possibly) an *a priori* belief that muscle histology or antibody profile may relate to prognosis, and thus may have biased the duration of treatment given to patients in this study. Therefore prospective, blinded, longitudinal studies are now required to confirm or refute these findings. Lastly, association cannot be confused with causality when considering MSA in JDM, and MSA may therefore be an epiphenomenon rather than directly pathogenic as is the case in many autoimmune diseases (with some notable exceptions).

Myositis Specific Antibody	**Target autoantigen**	**Known Frequency in JDM**	**Associated features**
Anti-TIF1g	TIF1g	23-32%	Ulcerative skin disease Lipodystrophy
Anti-NXP2	NXP2	13-23%	Younger age of disease onset Significant muscle weakness Calcinosis
Anti-MDA5	MDA5	6%	Cutaneous ulceration

			Arthritis Interstitial lung disease Minimal muscle disease
Anti-Mi2	NuRD complex	5%	"Classic dermatomyositis"
Antisynthetase Anti-Jo-1 Anti-PL-12 Anti-PL-7	Histidyl Alanyl Threonyl	<5%	Older age of disease onset Arthritis Interstitial lung disease
Anti-SRP	SRP	2-5%	Necrotizing myositis Cardiac involvement
anti-HMGCR	HMGCR	1%	No/minimal skin rash Myositis (characteristic muscle biopsy)
HMGCR = 3-hydroxy-3-methylglutaryl-coenzyme A reductase; MDA5 = melanoma differentiation-associated gene 5; NuRD = nucleosome remodeling-histone deacetylase; NXP2 = nuclear matrix protein 2; SRP= signal recognition particle; TIF1g = transcriptional intermediary factor 1-Gamma.			

Table 1.2. Myositis Specific Antibodies (MSA) in Juvenile dermatomyositis (JDM). *Target antigens, and associated clinical features.*

1.6 Aetiology of JDM

The exact aetiology of JDM is not known. The prevailing hypothesis is that the disease is the result of complex interactions between environmental factors and genetic susceptibility that lead to immune dysregulation (Wedderburn and Rider, 2009), in other words the same central hypothesis as for all complex autoimmune diseases. JDM is considered a composite multifactorial polygenic condition, in which the combination of specific genetic loci variations result in predisposition to developing JDM (Wedderburn and Rider, 2009). The development of international and multicentre collaborations along with the establishment of the Myositis Genetics Consortium (MYOGEN), an international genetic consortium, has helped towards that direction (Mendez *et al.*, 2003; McCann *et al.*, 2006; Mamyrova *et al.*, 2008).

1.6.1 Genetic risk factors

Several alleles in the human leukocyte antigen region (HLA) have been associated with an increased risk for several rheumatological diseases, including JDM. DRB1*0301, DQA1*0501, and HLA-B*08 have been demonstrated as contributing risk factors for the development of myositis in both children and adults of Caucasian descent (Pachman *et al.*, 1977; Mamyrova *et al.*, 2006; Wedderburn *et al.*, 2007b). HLA-DPB1*0101 and the DQA1*0301 allele serve as additional risk factors of myositis and JDM, respectively (Mamyrova *et al.*, 2006; Chinoy *et al.*, 2009). On the other hand, DQA1*0201, DQA1*0101, and DQA1*0 alleles may have a protective affect as they were found in lower frequency in children with JDM when compared to healthy controls (Mamyrova *et al.*, 2006). Polymorphisms in the pro-inflammatory cytokine

genes lnterleukin-1α (IL-1α) and Interleukin-1β (IL-1β) (Mamyrova *et al.*, 2008) and Tumour Necrosis Factor-α (TNF-α) have also been suggested as potential genetic risk factors for the development of childhood myositis (Pachman *et al.*, 2000; Mamyrova *et al.*, 2008). Moreover, TNF308A is known to be associated with the development of calcinosis and skin ulcerations (Pachman *et al.*, 2000). Independent variants of the HLA 8.1 haplotype, such as the R620W of the protein tyrosine phosphatase N22 (PTPN22), have also been highlighted as a genetic risk factors for myositis suggesting that numerous genetic regions are implicated in disease susceptibility (Chinoy *et al.*, 2008). Finally, children with JDM have been found to have decreased gene copy-number variations of complement C4 and resulting C4A deficiency has been demonstrated to be an important factor for increased genetic risk of JDM (Lintner *et al.*, 2016).

1.6.2 Environmental risk factors

The current knowledge on environmental risk factors for JDM is based solely on a few cohort and case control studies. Several infectious agents, including group A beta haemolytic streptococci (Martini *et al.*, 1992; Massa *et al.*, 2002), picornavirus (Bowles *et al.*, 1987; Pachman *et al.*, 1995) and enterovirus (Christensen *et al.*, 1986; Pachman *et al.*, 1995) have been studied with inconclusive results. Clusters of cases of JDM, reported in small studies, suggest that temporal and environmental factors may contribute to the pathogenesis of JDM (Symmons *et al.*, 1995). Study of specific subgroups of paediatric cases of inflammatory myopathies demonstrated a seasonal birth distribution, suggesting that perinatal

environmental factors may serve as risk factors for the development of JDM later in their life (Vegosen *et al.*, 2007). Other studied environmental factors include: exposure to ultraviolet light (Shah *et al.*, 2013b; Neely *et al.*, 2019), use of antibiotics (Pachman *et al.*, 2005), vaccinations (Limaye *et al.*, 2017), stressful events and dietary intake (Rider and Miller, 1997). Large epidemiological studies are needed to shed light on possible environmental risk factors for the development of JDM.

1.7 Treatment of Juvenile IIM

1.7.1 Consensus core set and response to treatment criteria

As childhood IIM are rare diseases, international collaboration is vital for clinical research, and to enable better understanding of mechanisms implicated in disease pathogenesis and more targeted treatments. For international collaboration to become possible, a number of core sets of variables have been proposed to enable collaborative research and allow integration of data between different centres in different countries. Recently, an optimal dataset containing 123 items in JDM have been published (McCann *et al.*, 2018). Moreover, the Paediatric Rheumatology International Trials Organization (PRINTO) and the International Myositis Assessment & Clinical Studies Group (IMACS) joined forces and defined response criteria to help clinicians quantify the clinical response and capture improvement in a homogeneous way and to facilitate future clinical trials (Rider *et al.*, 2017).

1.7.2 International consensus guidance for treatment of JDM

The choice and intensity of initial treatment for JDM is based on the presenting symptoms and disease severity (Stringer *et al.*, 2010). The presence of life-threatening weakness, major organ involvement and evidence of severe vasculopathic features including ulcerative skin lesions, gastrointestinal involvement and/or extensive calcinosis may influence the choice of treatment in JDM cases. Due to the disease rarity, limited randomised clinical trials exist to guide treatment decisions, and current therapeutic approaches are based on small case series and anecdotal experience. In an attempt to standardise treatment pathways, several therapeutic algorithms for the treatment of JDM have been developed based on expert consensus in North America and Europe (Huber *et al.*, 2010; Huber *et al.*, 2012; Enders *et al.*, 2017; Huber *et al.*, 2017; Kim *et al.*, 2017b). The Childhood Arthritis and Rheumatology Research Alliance (CARRA) have published Consensus Treatment Plans (CTP) for the initial treatment of JDM (Huber *et al.*, 2010), treatment after the first 3 months of disease onset (Huber *et al.*, 2012), treatment of cases with predominant skin disease (Kim *et al.*, 2017b) and cases with resistant to initial therapy skin disease (Huber *et al.*, 2017). Of note, these CTP have been developed via consensus methodologies, and do not represent therapeutic guidelines per se. Moreover, evidence-driven consensus-based recommendations for diagnosis and treatment of JDM have also been published as part of a European initiative called Single Hub and Access point for paediatric Rheumatology in Europe (SHARE) (Enders *et al.*, 2017).

1.7.3 Current therapeutic options

Currently available treatments have enabled treating clinicians to aim for induction and maintenance of complete disease remission in patients with JDM. When it comes to paediatric chronic diseases, desirable long-term outcomes further include normal growth and development, participation in all school and daily life activities and a good quality of life. Early pharmacological and non-pharmacological interventions in the form of a multidisciplinary treatment approach are therefore imperative for the prevention of irreversible soft tissue and organ damage and to secure the best long-term outcome. Pharmacological interventions for JDM include corticosteroids, disease-modifying anti-rheumatic drugs (DMARD), and biologic agents. Non-pharmacological interventions, such as physiotherapy, podiatry and occupational therapy and psychological support can enable patients to maintain their functional status by sustaining their muscle strength, stamina and their bone mineral density and advancing into adulthood with the less possible adverse effects on their physical and psychological health. JDM patients should be under the care of specialised tertiary centres where this combined multi-disciplinary approach is possible.

1.7.3.1 Corticosteroids

Since the introduction of corticosteroids as first line induction treatment of JDM, the overall prognosis of JDM has significantly improved (Fisler *et al.*, 2002). For the initial treatment of JDM, 2mg/kg/day of oral prednisolone, or pulses with 30mg/kg/day of intravenous (IV) methylprednisolone (MEP) followed by oral prednisolone are the two most commonly used corticosteroid regimes. When Seshadri *et al*. looked at the 3-year outcome in a comparative

study, no superiority was observed of one regime over the other, although IV MEP was used in the most severe cases (Seshadri *et al.*, 2008). In keeping with that, residual muscle weakness, relapsing disease and development of dystrophic calcification were less frequently observed in patients that received IV MEP compared to those that received oral treatment (Rider *et al.*, 1994; Romicka, 1995; Rider and Miller, 1997). Additionally, in a cost-effectiveness study published in 2000, IV MEP was superior in comparison to the oral regime (Klein-Gitelman *et al.*, 2000). Finally, there are specific circumstances where the use of IV MEP is further supported including the presence of dysphagia and gastrointestinal involvement where malabsorption is suspected (Rouster-Stevens *et al.*, 2008). Thus, many tertiary centres are now suggesting the use of IV MEP pulses for 3-5 days followed by a 2mg/kg/day dose of prednisolone (or IV MEP equivalent). Corticosteroid dose is usually given as a single morning dose, as this regimen has less effects on the hypothalamic-pituitary-adrenal function and growth, even in young children (Byron *et al.*, 1983). Regardless of the regimen used, corticosteroid dose is then tapered over a period of months depending on the response. Some cases of refractory childhood IIM such as anti-SRP antibody positive myositis may, however, require long-term treatment with a low dose of oral corticosteroids to prevent disease flare (Binns *et al.*, Submitted).

1.7.4 Disease-modifying anti rheumatic drugs (DMARD)

1.7.4.1 Methotrexate (MTX)

Methotrexate (MTX) has been used either as monotherapy or in combination with other drugs for the treatment of JDM for more than 30 years. The mechanism of action for

methotrexate is complex including inhibition of purine and pyrimidine synthesis, promotion of adenosine signalling, inhibition of transmethylation reactions, generation of reactive oxygen species, downregulation of adhesion-molecule expression, changes in cytokine profiles and suppression of eicosanoids and matrix metalloproteinases (Brown *et al.*, 2016). It is the most widely used DMARD used in JDM (Enders *et al.*, 2017). Despite the extensive use of MTX, most of the knowledge about its effectiveness in JDM was based mainly on case reports until relatively recently (Jacobs, 1977; Fischer *et al.*, 1979; Niakan *et al.*, 1980; Miller *et al.*, 1992; Ramanan *et al.*, 2005). In 2016, the results of the first randomised controlled trial (RCT) were published, where corticosteroid monotherapy was compared with the combined use of prednisolone/ MTX; or prednisolone/ ciclosporin. Prednisolone/ MTX was demonstrated to be the most efficacious regimen, with a median time to clinical remission of 42 weeks. Additionally, the combination of prednisolone and MTX was related with a lower adverse event rate when compared to the prednisolone/ ciclosporin group (Ruperto *et al.*, 2016). Additionally, early aggressive treatment with corticosteroids and MTX have been demonstrated to be efficacious for minimising long-term disease related complications, including calcinosis (Fisler *et al.*, 2002). A large retrospective study, looking in treatment practises in Europe and South America, demonstrated that MTX was the DMARD of choice in about 50% of paediatric cases in the studied regions (Guseinova *et al.*, 2011). Interestingly, in a case series where 8 children with mild to moderate JDM were included, MTX in combination with intravenous immunoglobulin were successfully used as first line treatment without corticosteroids (Levy *et al.*, 2010). In a recent survey performed in Germany and Austria where paediatric rheumatologists and neurology experts were asked regarding their

preferred therapeutic strategies, MTX was given for 24 months in approximately 50% of the cases (Hinze *et al.*, 2018). MTX is usually started at a dose of 15-20 mg/m^2/week and subcutaneous route is most commonly preferred (Enders *et al.*, 2017). Concomitant use of folic acid or folinic acid has been recommended in order to minimise the most commonly seen side effects associated with MTX use (Ravelli *et al.*, 1999), including gastrointestinal problems, bone marrow suppression and hepatotoxicity (Ortiz-Alvarez *et al.*, 2004; van der Meer *et al.*, 2007; Bulatovic *et al.*, 2011).

1.7.4.2 Ciclosporin

Ciclosporin is a calcineurin inhibitor that acts by inhibiting the transcription of IL-2, thereby blocking both the development of cytotoxic lymphocytes, and the proliferation of T helper cells (Lueck *et al.*, 1991). It has also been used in many centres (particularly in Europe) as a DMARD in JDM. Up to 2016, ciclosporin use had been mainly supported by efficacy data derived from small case series (Heckmatt *et al.*, 1989; Pistoia *et al.*, 1993; Reiff *et al.*, 1997). As discussed in the section above 1.7.4.1, in the only RCT comparing use of corticosteroids alone versus corticosteroids plus MTX or plus ciclosporin, prednisolone plus MTX or ciclosporin were found to have a better therapeutic effect compared to prednisolone monotherapy in patients with JDM, at 6 months and after at least 24 months of treatment (Ruperto *et al.*, 2016).Ciclosporin has several side effects including renal and liver toxicity, and bone marrow suppression (Fasano *et al.*, 2016), which in combination with the need of monitoring ciclosporin levels in the blood, makes ciclosporin use difficult in paediatric practice. Therefore, ciclosporin is usually reserved for refractory cases of JDM where other

immunosuppressants have failed. Of note, there have been cases of posterior reversible encephalopathy syndrome (PRES) in patients treated with ciclosporin, particularly when ciclosporin was combined with corticosteroids (Cosottini *et al.*, 2003; Dzudie *et al.*, 2009).

1.7.4.3 Mycophenolate Mofetil (MMF)

Mycophenolate mofetil (MMF) is increasingly used in refractory cases of JDM (Fasano *et al.*, 2016) and for cases complicated with ILD not responding to first line treatment (Kawasumi *et al.*, 2015), with promising results. MMF is metabolized in the liver in mycophenolic acid which selectively inhibits the de novo metabolism of purines with a direct effect in the proliferation of T and B cells, antibody production and leukocyte binding to endothelial cells (Rouster-Stevens *et al.*, 2010). A good response and safety profile of MMF have been shown in two retrospective studies where 12 (Dagher *et al.*, 2012) and 50 (Rouster-Stevens *et al.*, 2010) JDM patients were included, respectively. The commonest side effects are diarrhoea, bone marrow suppression, renal and liver toxicity (Castro and Gourley, 2012).

1.7.4.4 Azathioprine

Azathioprine is a derivative of mercaptopurine that inhibits purine metabolism resulting in the inhibition of DNA, RNA, and protein synthesis (Maltzman and Koretzky, 2003). It has been used in children with refractory JDM (Jacobs, 1977) where MTX has failed; or in cases where MTX cannot be tolerated. Because of the rarity of the disease, most of the knowledge on azathioprine comes from experience in adult patients (Dimachkie *et al.*, 2014), where azathioprine is the first choice of DMARD (Choy and Isenberg, 2002). Bone marrow

suppression and hepatotoxicity are the commonest side effects of azathioprine (Castro and Gourley, 2012), and routine blood monitoring is also recommended. Testing of thiopurine-methyl-transferase enzyme activity is recommended at initiation of treatment based on which subsequent adjustment of azathioprine dose may be needed (Dean, 2012). Further testing of azathioprine metabolites, 6-thioguanine and 6-methylmercaptopurine, has been used to help optimise effectiveness and safety of azathioprine in children with inflammatory bowel disease (Konidari *et al.*, 2014).

1.7.4.5 Cyclophosphamide (CyC)

Cyclophosphamide (CyC) is an alkylating agent that interferes with DNA replication and has a toxic effect to rapidly dividing cells (Donelli *et al.*, 1976). CyC is a third line therapeutic agent that may be used for severe and/or refractory JDM cases with significant organ involvement such as cardiac and pulmonary involvement, ulcerative skin disease, significant generalised subcutaneous oedema, gastrointestinal complications and the presence of significant vasculopathic complications, although there are no prospective trials of this treatment in JDM. In a retrospective review of 12 JDM cases with severe and refractory disease where CyC was used, 10 patients demonstrated significant improvement, both in muscular and extra-muscular manifestations in keeping with findings in adult patients with IIMs (Riley *et al.*, 2004; Kawasumi *et al.*, 2015). A subsequent study of 56 JDM patients treated with CyC compared with 144 patients not treated with CyC, using a marginal structural model approach, suggested significant improvement of overall disease activity in the CyC group at 6 months, 12 months and 24 months, with a good safety profile (Deakin *et al.*, 2018). This study, though,

had several limitations and results should be interpreted with caution; for example, confounding of the two treatment groups was not perfectly controlled for by the marginal structural model used for the analysis of the data, while full data on side effects were not available as they were not systematically collected. Alopecia, nausea, vomiting, haemorrhagic cystitis, infertility, teratogenicity and increased risk of infection and malignancy (Kawasumi *et al.*, 2015) are the main reported side effects. In boys of reproductive age, sperm banking should be considered; in girls of reproductive age, the use of triptorelin for ovarian protection has more recently been recommended (Brunner *et al.*, 2015), but the evidence is limited and there is a theoretical risk of inadvertently actually increasing ovarian toxicity with this approach.

1.7.4.6 Hydroxychloroquine (HCQ)

Hydroxychloroquine (HCQ) is an antimalarial medication that reduces the formulation of peptide-MHC protein complexes necessary to stimulate CD4+ T cells resulting in down-regulation of the immune response against autoantigens (Fox, 1993). HCQ is mainly used in less severe cases of JDM with mild skin disease, based on limited evidence from anecdotal experience and two small reviews (Olson and Lindsley, 1989; Sterba and Wahezi, 2014). Although HCQ is thought be a very safe medication, ocular toxicity has been recognised as a possible side effect. Thus, a baseline examination is recommended ideally within 6 months of starting therapy and then patients should be referred for annual screening after 5 years of therapy as per the Royal College of Ophthalmologists guidelines (Ophthalmologists, 2018).

1.7.4.7 Tacrolimus

Tacrolimus is a macrolide antibiotic that forms a complex with FK506 binding protein which then inhibits calcineurin, reducing the transcription of IL-2, IL-3, IL-4, TNF-α, and activation of T cells (Andersson *et al.*, 1992; Mok *et al.*, 2005). Evidence for the use of tacrolimus in children with JDM originates from small case series (Hassan *et al.*, 2008; Huber, 2009). There is increasing evidence for the use of tacrolimus as adjunctive to conventional treatment for DM associated ILD in adults (Kurita *et al.*, 2015; Witt *et al.*, 2016) but its use in paediatric cases needs to be further investigated.

1.7.4.8 Intravenous immunoglobulin (IVIG)

Intravenous immunoglobulin (IVIG) has several anti-inflammatory and immunomodulatory effects with different mechanism of action being proposed in different diseases (Gelfand, 2012). In JDM, IVIG is thought to downregulate complement activity and deposition of membrane attack complex (MAC) on capillaries and muscle fibres, reduce the expression of adhesion molecules, and cytokine production and alter pro-inflammatory molecule gene expression in the affected muscles (Gelfand, 2012). IVIG is recommended in JDM patients refractory to first line treatment, although the evidence is derived mainly from case reports and small case series (Lam *et al.*, 2011). The first reports of IVIG use in JDM patients go back to the 1980s, and suggested significant improvement in muscle and skin disease, and reduction in cumulative corticosteroid dose used (Lang *et al.*, 1991; Collet *et al.*, 1994; Sansome and Dubowitz, 1995; Al-Mayouf *et al.*, 2000). In the largest retrospective study published in 2011, 30 patients treated with IVIG were shown to have lower disease activity

up to 4 years post-diagnosis when compared to 48 IVIG naïve patients, especially for those patients that had steroid resistant disease (Lam *et al.*, 2011). Both the CARRA and SHARE guidance proposed the use of IVIG in refractory JDM once conventional treatment with steroids and methotrexate has failed or early in disease course in the most severe cases (Mimori *et al.*, 2007; Kim *et al.*, 2017b). IVIG related adverse effects are usually mild and resolve after discontinuation of the medication. The immediate adverse events include flu-like symptoms (malaise, fever, rigors, myalgia), headache, chest tightness, fatigue, dyspnoea, back pain, nausea, vomiting, hyper- or hypotension, tachycardia, and anaphylactic or anaphylactoid reactions. Anaphylactic reactions are more frequently seen in patients with significant IgA deficiency and those with anti-IgA antibodies of the IgE type (Burks *et al.*, 1986; Ferreira *et al.*, 1988). Acute renal failure, thromboembolic events, aseptic meningitis, neutropenia, and occasionally arthritis are rare but have been reported as adverse events (Orbach *et al.*, 2005). Recently, the use of subcutaneous immunoglobulin has been reported to be effective in a JDM case with systemic reaction to IVIG (de Inocencio *et al.*, 2016). Reports in adults further support that treatment with subcutaneous immunoglobulin may represent a promising cost-effective alternative to IVIG (Danieli *et al.*, 2011; Abolhassani *et al.*, 2012; Gelardi *et al.*, 2014; Hachulla *et al.*, 2017).

1.7.5 Biologic agents

1.7.5.1 Targeting B-cells

1.7.5.1.1 Rituximab (RTX)

Rituximab (RTX), a chimeric monoclonal antibody directed against the human CD20 receptor. RTX is the most widely used biologic agent in IIM and is thought to act primarily by depleting CD20-positive B cells (Oddis *et al.*, 2013). Case reports and case series in adults and children with JDM have suggested that rituximab may be an effective treatment in refractory cases (Cooper *et al.*, 2007; Bader-Meunier *et al.*, 2011; Chiu and Co, 2011), although no improvement was observed in calcinosis. In 2013, results of a randomized control trial that included 152 adults with myositis, and 48 children with JDM were published. Interestingly, 83% of the included patients met the definition of improvement on rituximab, although the primary end point was not met, mainly due to the study design; children demonstrated a better response when compared to adult patients (Oddis *et al.*, 2013). RTX might have a role in the treatment of anti-SRP positive myositis with studies suggesting improvement in muscle disease and in muscle enzymes with RTX treatment (Valiyil *et al.*, 2010; Luca *et al.*, 2012; Binns *et al.*, 2017). RTX is also emerging as a promising treatment for rapidly progressive ILD in patients with anti-MDA5 positive myositis (Watanabe *et al.*, 2016; Koichi *et al.*, 2017; Tokunaga and Hagino, 2017; So *et al.*, 2018). Intravenous MEP, chlorphenamine and paracetamol are recommended an hour prior to RTX infusion in an attempt to minimize both early and late onset reactions, including fever, rash, headache, itchiness, back pain and severe allergic reactions (Chiu and Co, 2011). Hypogammaglobulinaemia and late onset neutropenia

have also been reported after treatment with RTX predisposing those patients to an increased risk of infections (Salmon *et al.*, 2015).

1.7.5.2 Anti-Tumour Necrosis Factor (TNF) agents

1.7.5.2.1 Infliximab and Adalimumab

Upregulation of TNF-α and TNF receptor has been observed in muscle tissue of patients with IIM while there is increased expression of TNF in the endothelium of patients with DM (Efthimiou, 2006). Based on these findings, a possible role of anti-TNF biologic agents in JDM has been suggested. Infliximab, a chimeric monoclonal antibody, and adalimumab, a recombinant monoclonal antibody, bind to human TNF-α, thereby interfere with binding to receptor sites and subsequent cytokine-driven inflammatory processes (Hu *et al.*, 2013). In 2008, Riley *et al*. reported 5 cases of refractory JDM and development of calcinosis successfully treated with infliximab (Riley *et al.*, 2008). In 2016, Campanilho-Marques et al. reported results of 66 JDM patients recruited from the UK actively treated with anti-TNF agents, infliximab or adalimumab, with significant improvements in overall disease activity including muscle and skin involvement (Campanilho-Marques *et al.*, 2016b). In contrast, etanercept, a recombinant DNA-derived protein composed of TNF receptor linked to the Fc portion of human IgG1, was not shown to be efficacious in two prospective case studies for adult DM and JDM, respectively (Iannone *et al.*, 2006; Rouster-Stevens *et al.*, 2014). A paradoxical effect has been reported in adults with DM treated with infliximab; there was upregulation of type I interferon (IFN) activity associated not only with lack of clinical response but also with disease exacerbation (Dastmalchi *et al.*, 2008). Moreover, in a recent

systematic literature review, 20 patients were described where new onset of DM/PM was recorded as a result of anti-TNF treatment for other autoimmune diseases (Brunasso *et al.*, 2014). This could be the result of the increased IFN-γ production after treatment with anti-TNF agents (Brunasso *et al.*, 2010); IFN-γ has been found to be increased in muscle tissue of DM patients (Giris *et al.*, 2017). The exact mechanism is not yet understood.

1.7.5.3 Other biologic agents

There are increasing reports of the use of other biologic agents in dermatomyositis/polymyositis, mainly in adult patients. A recently published review of current pharmacological treatment for idiopathic inflammatory myopathies (Fasano *et al.*, 2016) described three cases of refractory myositis treated with tocilizumab, an IL-6 receptor antagonist, with promising results. Abatacept, a soluble fusion protein comprising the extracellular domain of human cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and a fragment of the Fc domain of human immunoglobulin G1, has been reported to be effective in a recalcitrant JDM case with ulceration and calcinosis (Arabshahi *et al.*, 2012). Abatacept inhibits T-cell activation by binding to CD80 and CD86 on antigen presenting cells, thus blocking the required CD28 interaction between antigen presenting cells and T-cells (Tjarnlund *et al.*, 2018). In a phase-IIb randomised delayed start clinical trial, in which 9 DM and PM adult patients were treated with abatacept, lower disease activity was observed in nearly half of the included patients (Tjarnlund *et al.*, 2018) with an acceptable safety profile. A number of other biologic therapies have been used in adults with IIM, including sifalimumab (an anti-IFN-α monoclonal antibody), alemtuzumab (a humanized anti-CD52 IgG1 monoclonal

antibody), eculizumab (a humanized anti-C5 IgG2/4 monoclonal antibody) and basiliximab (a chimeric anti-IL-2 receptor monoclonal antibody), but none of them has been ever used in children (Moghadam-Kia *et al.*, 2017).

1.7.6 Adjunctive therapies

In addition to the therapeutic regimes described above, a number of other pharmacological and non-pharmacological treatments are used in patients with JDM. High factor sunscreen is recommended to be used on a daily basis to prevent disease flares from UV light exposure (Enders *et al.*, 2017). Calcium and vitamin D supplements are used to prevent development of osteoporosis resulting from immobility and long-term corticosteroid use (Soybilgic *et al.*, 2014; Vojinovic and Cimaz, 2015). The use of gastroprotective medications is suggested along with mainstream treatment to prevent gastrointestinal ulceration, bleeding and ultimately perforation associated with corticosteroids use (Narum *et al.*, 2014).

Physiotherapy and occupational therapy are important adjunctive measures. Children with IIM are usually deconditioned and have reduced functional ability. They often experience significant fatigue (Takken *et al.*, 2005) and are unable to perform aerobic and anaerobic exercises (Butbul Aviel *et al.*, 2011). Moreover, decreased physical activity along with corticosteroid treatment increase the risk of obesity, development of diabetes, and in the longer term, cardiovascular disease (an area that will be considered in much more detail later in this thesis). A specialized physiotherapy program is essential to prevent development of joint contractures, and to re-build muscle strength. Several studies have demonstrated the

benefit of a specialized physiotherapy program in increasing muscle strength and improving muscle endurance (Maillard *et al.*, 2005; Habers *et al.*, 2012).

Despite the increased knowledge about the pathogenesis of JDM and availability of different treatments, the outcome of JDM still varies significantly from patient to patient. As vasculopathy is central to the pathogenesis of JDM understanding the underlying mechanisms and developing ways of monitoring the vasculopathy may help develop new therapeutic approaches.

1.8 The vasculopathy of JDM

The microvasculature composes of vessels with a diameter < 300μm which includes arterioles, capillaries and venules (Pries *et al.*, 1990). Vasculopathy refers to the non-inflammatory changes of the microvasculature mainly caused by immune complexes deposition and micro-thrombosis; in contrast, vasculitis mainly refers to an inflammatory process characterized by leukocytic infiltration and fibrinoid changes of the vascular wall (Savage and Ng, 1986). Microvascular alterations are hallmarks of several connective tissue diseases including JDM, which is characterised by both vasculopathic and vasculitic features (Banker and Victor, 1966).

Vasculopathy is suggested to play a central role in the pathogenesis of muscle and cutaneous involvement (Whitaker and Engel, 1972; Bohan and Peter, 1975) in children with JDM. Vasculopathy has also a central role in the development of other severe systemic features of the disease: intestinal ischaemia and perforation, interstitial lung disease and fibrosis,

dystrophic calcification, CNS, and cardiac involvement (Crowe *et al.*, 1982; McCann *et al.*, 2006). Thus, development and persistence of vasculopathy contributes significantly to the burden of JDM in children, and long-term prognosis. The exact nature of vasculopathy remains unclear, and probably demonstrates a dynamic effect as it seems to differ at different stages of the disease.

Early on, at (or close to) disease onset, there is evidence of a true inflammatory small vessel vasculitis driven by interferons and other cytokines (Whitaker and Engel, 1972; Baechler *et al.*, 2007). Characteristic muscular findings include the presence of inflammatory changes in the proximity of perimysial arteries along with microinfarcts, perifascicular atrophy and ischaemic vacuoles in the muscle fibres. Immunoglobulin and complement have been identified in the vessel walls of skeletal muscle in children with JDM (Whitaker and Engel, 1972). Complement deposition of the terminal C5b-9 membrane attack complex (MAC) is seen in lesional muscle biopsies, suggestive of an immune-mediated small vessel vasculitis (Whitaker and Engel, 1972; Kissel *et al.*, 1986) (**Figure 1.10**). A hypothesis for more than 20 years has been that circulating antibodies against endothelial antigens damage blood vessels; the resulting injury then leads to complement deposition which results in ischaemic myofiber injury visible as perifascicular atrophy and infarction. Activated MAC then triggers endothelial cells to produce a number of proinflammatory cytokines (Lundberg *et al.*, 1997) which further promote the inflammatory process through a positive feedback loop that upregulates endothelial intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Sallum *et al.*, 2004). Moreover, activated MAC and subsequently endothelium

results in B-cell, CD4+ T-cell, and plasmacytoid dendritic cell infiltration with an important later effect on angiogenesis (Dalakas, 2015; Findlay *et al.*, 2015). Activated dendritic cells lead to increased type I IFN production. This is demonstrated by increased levels of type I IFN and upregulation of type I IFN related gene expression, including the IFN stimulated gene 15 (ISG-15) and a type 1 interferon inducible antiviral molecule (MxA), in the plasma and muscle biopsies (Baechler *et al.*, 2007; Walsh *et al.*, 2007). Type I IFN have a number of different biological effects, including impaired myotube differentiation (Franzi *et al.*, 2013), upregulation of major histocompatibility complex (MHC) class I and class II resulting in activation of endoplasmic reticulum stress response in DM (Nagaraju *et al.*, 2000), and upregulation of adhesion molecules promoting T-cell migration (Wedgwood *et al.*, 1988; Fall *et al.*, 2005; Baechler *et al.*, 2007; Eloranta *et al.*, 2007; Lopez de Padilla *et al.*, 2007), such as macrophage inflammatory protein (MIP), monocyte chemoattractant protein (MCP)-1, and MCP-2. T-cells are further involved in the disease process through lesional T helper type 17 (Th17) cells, which further affect IL-6 and IL-17 production that relate to the interferon response and disease activity. Indeed, type I IFNs are increasingly recognised to play a central role into the pathogenesis of JDM and constitute determinants of the associated vasculopathy (Baechler *et al.*, 2007).

Later in the course of JDM, endothelial cell swelling, necrosis and luminal occlusion of capillaries and arterioles (Crowe *et al.*, 1982; Emslie-Smith and Engel, 1990) are characteristic vasculopathic features of the disease. Impaired repair mechanisms subsequently lead to loss of the capillary network (Fall *et al.*, 2005). Capillary drop-out is a commonly and well reported

observation in JDM, as it comprises the major vasculopathic feature detected in muscle biopsies and in the skin (Emslie-Smith and Engel, 1990; Miles *et al.*, 2007; Wedderburn *et al.*, 2007c; Christen-Zaech *et al.*, 2008). The reason for the impaired blood vessel repair is not fully understood. A number of different cytokines and chemokines, such as IL-1, IFN-α, and IFN-γ, have angiostatic effects on endothelial cells (Koch, 1998). Moreover, the IFN induced angiostatic CXC chemokines that lack the 3–amino acid sequence Glu-Leu-Arg (the ELR motif), including CXCL9, CXCL10 and CXCL11, have been found to be elevated in muscle biopsies (Fall *et al.*, 2005) and serum of untreated JDM patients (Fall *et al.*, 2005; Baechler *et al.*, 2007), correlating with the degree of capillary loss, mononuclear cell infiltration and strongly to the vasculopathy of JDM. Neovascularization has been suggested to take place later in the disease process (Nagaraju *et al.*, 2006) and in response to treatment, suggesting that the vasculopathy of JDM is a dynamic process.

Occlusive vasculopathy is the main risk factor for disease chronicity having a major impact in the development of the severe complications of JDM including: skin ulceration, gastrointestinal vasculitis causing intestinal ischaemia and ultimately gut infarction and cardiopulmonary involvement (Pachman *et al.*, 1998; Mamyrova *et al.*, 2007; Gitiaux *et al.*, 2016). It is also possible that occlusive vasculopathy contributes to chronic subcutaneous calcinosis, which occurs in up to 34% of cases (McCann *et al.*, 2006; Marhaug *et al.*, 2008). Whilst in general terms it is believed that corticosteroids are effective for the treatment of true vasculitis in the early stages of JDM, it is suggested that corticosteroids may potentially worsen the occlusive vasculopathy that occurs in the later stages of the disease (Bukulmez *et*

al., 2001). This observation emphasises the importance of better understanding the pathogenesis of the vasculopathy of JDM, to use existing medications judiciously at different disease stages, to facilitate the development of novel biomarkers to guide treatment, and ultimately aim for alternative, more targeted and individualised therapeutic approaches.

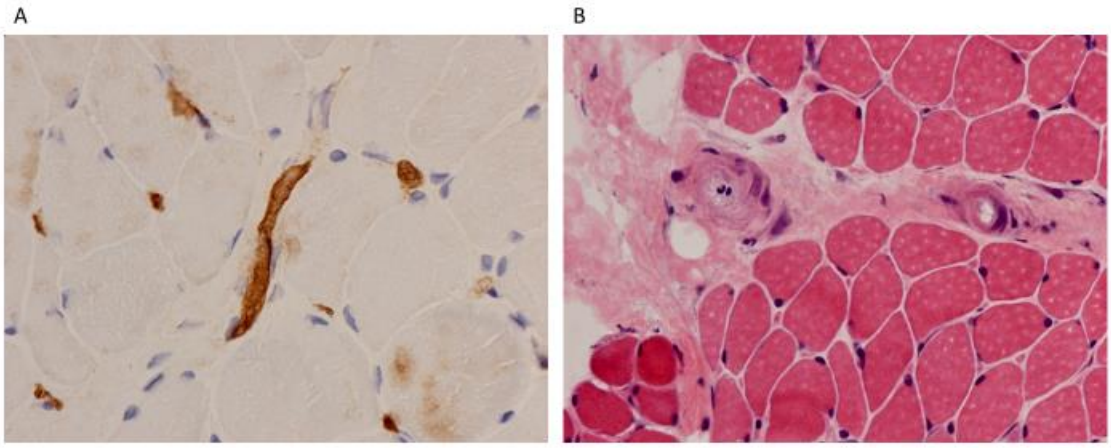

Figure 1.10. Muscle biopsy findings in a patient with Juvenile dermatomyositis (JDM). *(A) Haematoxylin and eosin (H&E) stain showing vessel endothelial swelling. (B) Immunohistochemical staining for membrane attack complex (MAC) showing deposition on muscle capillaries. Original magnification 40x.*

1.9 Non-invasive detection of vascular injury in JDM

Endothelial injury is thought to be an early event in the development of vascular disease in JDM. Although, the endothelium was initially thought to be a simple layer covering the vascular tree, with no specific functions other than a physical layer between blood and the underlying tissues with pre-determined permeability to water and electrolytes (Rajendran *et al.*, 2013), it is now well recognised that endothelium plays an important role in vascular

homeostasis through regulation of vascular tone and growth, leukocyte and platelet interaction, and maintenance of the balance between thrombotic and anticoagulant properties of the blood (Verhamme and Hoylaerts, 2006).

Endothelial activation and injury is characterised by enhanced production of the vasomotor factors, overexpression of adhesion molecules and selectins, overproduction of proinflammatory cytokines and chemokines and alteration of thrombotic pathways (Pober *et al.*, 2009). Significant injury subsequently leads to endothelial cell desquamation from the vessel wall resulting in entire endothelial cells to be shed into the circulation. Injured endothelial cells may also degrade their cell surface heparan moieties, and may release membrane vesicles known as endothelial microparticles (EMP) (Brogan and Dillon, 2004; Brogan *et al.*, 2004; Pober *et al.*, 2009).

One major hurdle to the study and detection of the vasculopathy of JDM has been a lack of non-invasive biomarkers. Tissue biopsy, and/or arteriography, extensively used in systemic vasculitides for the detection of vascular injury (Brogan *et al.*, 2002; Brogan *et al.*, 2010), are too invasive to be used repetitively for regular monitoring and follow-up of children with JDM. There has been increasing interest in plasma biomarkers of endothelial dysfunction, with efforts mainly focusing on soluble cellular adhesion molecules, including ICAM-1, VCAM-1, E-selectin and others (Hwang *et al.*, 1997; Ridker *et al.*, 1998) which are typically found on the surface of endothelial cells in response to activation. Despite the extensive study of these molecules, their prognostic role remains uncertain. Brogan *et al*., described two methods for

detecting endothelial cell components in blood which allow non-invasive assessment of vascular injury: circulating endothelial cells (CEC), and EMP (Brogan and Dillon, 2004; Brogan *et al.*, 2004; Clarke *et al.*, 2008; Clarke *et al.*, 2010; Eleftheriou *et al.*, 2011; Eleftheriou *et al.*, 2012). CEC and EMP could therefore serve as non-invasive biomarkers for the detection of endothelial injury, monitoring of response to treatment and prediction of disease activity trajectories relating to persistent endothelial injury in JDM. Virtually nothing is known about these indices in JDM, however. A background into the development of these biomarkers is provided in individual chapters.

1.10 Accelerated atherosclerosis in JDM

Atherosclerosis is considered a chronic inflammatory process. Circulating leukocytes, when attached to the endothelial layer, initiate the atherogenic processes ultimately leading to the development of atherosclerotic plaques (Singh *et al.*, 2002). Atherosclerosis is known to begin early in childhood with reports suggesting a decrease in aortic distensibility, starting at 2.3 years of age (Voges *et al.*, 2012). Accelerated atherosclerosis has been shown to be an important determining factor for the mortality and morbidity in patients with autoimmune diseases (Esdaile *et al.*, 2001; Meune *et al.*, 2010). A recent study comparing patients with rheumatoid arthritis (RA) and patients with diabetes found equal frequency of atherosclerosis between the two diseases (Stamatelopoulos *et al.*, 2009). Endothelial dysfunction has been demonstrated as a key step in the initiation and maintenance of atherosclerosis (Steyers and Miller, 2014) suggesting that endothelial dysfunction may be the link mechanism between chronic inflammatory diseases and atherosclerosis. Increased circulating inflammatory

cytokines and chemokines, autoantibodies targeting endothelium (directly, or indirectly via immune complex deposition) may work in concert with traditional risk factors to drive cardiovascular disease, which subsequently leads to dysregulation of endothelial pathologic vaso-relaxation, enhanced leukocyte adhesion to endothelium, impaired endothelial permeability, and a prothrombotic state/activated endothelial state (Steyers and Miller, 2014). It has thus become evident that endothelial dysfunction can be used as an early indicator of atherosclerotic disease before the development of structural arterial changes, and has been suggested as a possible predictor of future cardiovascular events (Nozaki *et al.*, 2009; Yeboah *et al.*, 2009).

Regardless of the exact nature of the small vessel vasculopathy in JDM, in the longer term chronic endothelial injury may result in structural arterial changes affecting both peripheral and central arteries, ultimately leading to accelerated atherosclerosis and premature cardiovascular morbidity later in adulthood. Several risk factors could contribute towards that: the chronic and inherent vasculopathic nature of the disease; chronic systemic inflammation; and the presence of traditional cardiovascular risk factors that are more frequently observed in JDM including hypertension, dyslipidaemia and diabetes (Svenungsson *et al.*, 2001; Solomon *et al.*, 2006). In support of this, adolescent patients with JDM have higher risk of cardiovascular and cerebrovascular disease, including atherosclerosis, transient ischaemic attacks and cerebral infarction, in a large retrospective study (Silverberg *et al.*, 2018). Similarly, when 8 adults with a history of JDM were compared with 8 healthy controls in a small pilot study, they were found to have evidence of accelerated atherosclerosis as

demonstrated by increased carotid-intima-media thickness (CIMT) and altered flow-mediated dilatation (FMD) (Eimer *et al.*, 2011). It is thus possible that the combination of chronic endothelial injury caused by persistent small vessel vasculitis, chronic systemic inflammation, long-term corticosteroid use, sedentary lifestyle and conventional cardiovascular risk factors predispose patients with JDM to early atherosclerosis. This altered risk of cardiovascular diseases may be reflected by endothelial dysfunction, even in the absence of detectable atherosclerosis. The study of endothelium and its dysfunction may, thus, shed light on pathophysiological mechanisms that drive accelerated atherosclerosis in this population, early detection of which is essential for the development of preventative strategies able to reduce the risk of accelerated cardiovascular morbidity before these patients progress into adulthood.

1.11 Hypothesis and aims

This thesis was based on the following **hypotheses**:

(a) The vasculopathy of JDM begins as an inflammatory small vessel vasculitis driven by pro-inflammatory cytokines (including IFNs and IFN regulated cytokines/chemokines) that target the endothelium;

(b) As a consequence of vasculitis and systemic inflammation, circulating microparticles (MP) contribute to occlusive vasculopathy by promoting intravascular small vessel thrombin generation;

(c) Structural medium-sized arterial changes indicative of premature atherosclerosis will develop as a consequence of endothelial injury and prothrombotic processes.

The specific **aims** of this thesis were to:

(i) Examine whether biomarkers of endothelial injury, subclinical inflammation and hypercoagulability relate to clinical disease activity, treatment and development of calcinosis in a large cohort of JDM patients.

(ii) Establish whether structural arterial changes and endothelial dysfunction indicative of premature atherosclerosis may develop as a result of JDM and its related vasculopathy.

2 Methods and materials

General methods used in this thesis are described in this chapter. More detailed methods are provided in the individual chapters where applicable.

2.1 Subjects

2.1.1 Study design and patient population

Patients aged 2-19 years old with JDM as defined by Peter and Bohan criteria (Bohan and Peter, 1975) were recruited from Great Ormond Street Hospital NHS Foundation Trust (GOSH) through the Juvenile Dermatomyositis Cohort and Biomarker Study (JDCBS) (McCann *et al.*, 2006; Martin *et al.*, 2011). The JDCBS is a national registry for children with IIM based at University College London (UCL) Great Ormond Street Institute of Child Health, launched in 2000 (Martin *et al.*, 2011). To date 589 children have been recruited from 16 participating centres across the UK. At GOSH, patients were recruited through the outpatient JDM clinics and through inpatient ward admissions. Informed consent was obtained from all parents/guardians and age appropriate assent with local ethical approval (MREC 1/3/022).

Patients with non-JDM associated co-morbidity that could cause endothelial injury (sickle cell disease, type 1 diabetes mellitus, chronic kidney failure, chronic infection, or malignancy) were excluded from the study.

Two study designs were used:

Study 1 was a cross-sectional study that examined biomarkers of endothelial injury in patients already classified as having active JDM compared to those with inactive JDM and healthy controls.

Study 2 was a prospective study that examined endothelial biomarker changes over time in patients with JDM (studied longitudinally at recruitment and latest follow-up appointment).

2.1.2 Healthy controls

Biomarkers of endothelial injury were also studied in age-similar and sex matched healthy controls recruited to two other major projects examining endothelial injury biomarkers in systemic vasculitis and stroke in children: 1. An action medical research fellowship to Dr Eleftheriou (Eleftheriou *et al.*, 2012); 2. A Kawasaki disease study funded by the British Heart Foundation led by PA Brogan (Shah *et al.*, 2015). Additional controls were recruited through the Versus Arthritis (formerly known as Arthritis Research UK) Centre for adolescent rheumatology young scientist days, where adolescents were invited to spend a day in the lab, and donate blood with informed written consent. Healthy volunteers recruited as controls through these young scientist days or within staff at UCL were approached if they had no medical history of acute or chronic illnesses and specifically no symptoms of intercurrent infection at time of sampling. Informed consent was obtained from all adolescent participants with local ethical approval (REC 11/LO/0330).

2.1.3 Clinical data collection and disease activity measures

The following information was collected for all study participants: age, sex, race/ethnicity, height, weight, body mass index, blood pressure, age of disease onset, age at diagnosis, disease duration at recruitment, family history of cardiovascular disease, smoking status, histopathology scores from muscle biopsy performed at diagnosis. Laboratory test results were documented for: haemoglobin (Hb), platelets (PLT), white cell count (WCC), neutrophils, lymphocytes, cholesterol and triglycerides, antinuclear antibody (ANA) status and MSA, clinical manifestations, and treatment regimens used. Testing of MSA was performed at Bath Institute for Rheumatic Diseases using a combination of immunoprecipitation and ELISA (Tansley et al., 2017). The following MSA were screened: anti-TIF1g, anti-OJ, anti-EJ, anti-PL12, anti-PL7, anti-SRP, anti-Jo1, anti-Pm-Scl75, anti-Pm-Scl100, anti-Ku, anti-SAE, anti-NXP2, anti-MDA5, anti-Mi2, and anti-Ro52.

Different validated tools and indices already used in the JDCBS cohort were employed to capture the extent of disease activity. These were:

1. The childhood Myositis Assessment Scale (CMAS). CMAS is a quantitative, non-invasive measure of physical function, muscle strength and stamina used in children with IIM. It was first published in 1999, and validation studies have been performed (Lovell *et al.*, 1999; Huber *et al.*, 2004). It comprises a 14-item instrument. When all scores are added the total sum ranges from 0 (very poor physical function and strength) to 52 (normal physical function and strength). Physicians usually need 10-15 minutes to perform the whole assessment. This tool is provided in **Appendix 2**.

2. Manual Muscle Testing -8 (MMT-8). MMT-8 is a measure of muscle strength which assesses the strength of eight muscle groups (neck flexors, deltoid, biceps, gluteus maximus, gluteus medius, quadriceps, wrist extensors and ankle dorsiflexors) (Rider *et al.*, 2010). Each muscle group is evaluated using Kendall's 0 –10 point scale (Harris-Love *et al.*, 2009); scores are then added, to provide a total ranging from 0/80 (very weak), to 80/80 (normal muscle strength). The tool is provided in **Appendix 3**.

3. Laboratory markers of disease activity. The following laboratory markers of disease activity were collected: erythrocyte sedimentation rate (ESR, reference range, RR < 10 mm/h), C-reactive protein (CRP, RR < 20 mg/l), creatine kinase (CK, RR 6–330 U/l), alanine aminotransferase (ALT, RR 10-35 U/l), aspartate aminotransferase (AST, RR 15-50 U/l) and lactate dehydrogenase (LDH, RR 450–770 U/l). Serum levels of muscle enzymes have been traditionally used as markers of myositis activity. LDH, when combined with AST, has been suggested to represent a good predictor of myositis flare in JDM (Guzman *et al.*, 1994).

4. Physician Global Assessment (PhyGLOVAS) of disease activity using a 10-cm visual analogue scale (VAS). The treating physician rated their subjective impression of the overall disease activity using a 10-cm VAS, with 0 corresponding to no disease activity and 10 to maximum disease activity (Rider *et al.*, 1997).

5. Modified Skin Disease activity score (DAS). This is a 5-point skin DAS, adapted for retrospective studies, wherein a higher score corresponds to worse skin disease activity (Lam *et al.*, 2011). The JDM modified skin DAS assesses the degree of skin involvement and the presence of vasculopathic manifestations. It gives a score ranging from 0 to 5. The following items are scored in the modified skin DAS:

- Erythema (2 points if present).
- Gottron's papules (1 point if present).
- Heliotrope rash (1 point if present).
- Vasculitis (1 point if present)—includes abnormalities in nailfold capillaries.

This skin DAS has been validated and has been found to have good interrater agreement with low variability, and good internal consistency (Bode *et al.*, 2003; Campanilho-Marques *et al.*, 2016a).

6. Functional ability through the Childhood Health Assessment Questionnaire (CHAQ). The CHAQ assesses the ability of the child to perform daily activities (Singh *et al.*, 1994). The CHAQ correlates better with measures of muscle strength than muscle enzymes, and responds well to clinically significant changes (Feldman *et al.*, 1995; Huber *et al.*, 2001). A copy of the CHAQ is provided in **Appendix 4**.

7. Parent's global assessment of the patient's overall well-being on a 10 cm VAS (Par GLOVAS); the parent of the child with JDM and/or the child (if aged appropriately) make a global assessment of the child's overall wellbeing on a 10 cm VAS.

2.1.4 Patient groups

A modified definition of the PRINTO criteria for inactive JDM was used (Lazarevic *et al.*, 2013). Patients were classified as having inactive JDM in the absence of skin disease at time of assessment, and 3 at least of the following 4 criteria:

1. CK ≤ 150 U/L,

2. CMAS ≥ 48/52,
3. MMT -8 ≥ 78/80, and
4. PhyGLOVAS ≤ 0.2/10.

I chose this approach as a recent study (Almeida *et al.*, 2015) demonstrated that the PRINTO criteria for JDM clinical inactive disease (Lazarevic *et al.*, 2013) are mainly weighted towards muscle disease, and in some cases skin disease is disregarded. To account for that I modified the PRINTO definition to include the absence of skin disease as a mandatory criterion for the definition of clinically inactive disease in my cohort.

2.2 Materials

2.2.1 Reagents for blood collection

The following samples were collected from JDM patients and healthy controls: 1.4ml of 3.2% trisodium citrate (Sartsedt), 1.2ml of Ethylenediaminetetraacetic acid (EDTA; Sartsedt), 1.2ml of 5 ml polypropylene containing no anticoagulant (Serum Z; Sartsedt). Sterile 20 ml universal bottles containing 40µl of preservative free heparin (monoparin, CP pharmaceuticals Ltd, 1000 U/ml) were also collected when Peripheral Blood Mononuclear cell (PBMC) isolation was needed.

2.2.2 Preparation of platelet poor plasma

Blood from patients/child controls and adult healthy volunteers was collected in 3.2% buffered citrate. Samples were transferred to aliquots and centrifuged at 5000 g for 5 min

twice to obtain platelet poor plasma (PPP). PPP samples were divided into aliquots and stored at –80°C for future batch testing.

2.2.3 Preparation of serum

Blood from patients/healthy controls and adult healthy volunteers was collected in 5 ml polypropylene containing no anticoagulant tubes. Samples were left at room temperature for 2 to 4 hours and they were then centrifuged at 2500 g for 10 min to obtain serum. Serum samples were divided into aliquots and stored at –80°C for future batch testing.

2.2.4 Isolation and enumeration of CEC with immunomagnetic bead extraction

2.2.4.1 Bead based extraction reagents

The following reagents were used:

- DYNAL bead technology (Invitrogen) was used for isolation of CEC: Dynabead Pan mouse IgG were labelled with an unconjugated IgG1 antibody to CD146, clone s-endo1 (Biocytex).
- The Dynal MCP-L magnet which holds 1-8 ml tubes was also used.
- Phosphate buffered saline (PBS; Sigma).
- 0.1% bovine serum albumin (BSA; Sigma).
- PBS containing 0.1% BSA and 0.6% sodium citrate (PBAC).

2.2.4.2 Preparation of immunomagnetic beads

A total of 350 µl of beads (Dynal M450 pan mouse IgG beads) were added to a 5ml polypropylene tube. The tube used was then placed into the magnet (MPC-L; Dynal Biotech) for 2 minutes allowing the beads to attach to the wall of the tube. The supernatant was then carefully discarded, and the remained beads were re-suspended in 1ml PBS with 0.1% BSA added. The tube was placed in the magnet again and the supernatant discarded as above two further times. The beads were finally re-suspended in 950 µl of PBS (with 0.1% BSA) and 400 µl of s-endo-1 antibody (Biocytex) were added (final concentration of 25 µg/ml). The tube was sealed, and the sample mixed on a roller mixer for 2 hours at 4^{o}C. Beads were washed 3 times as before and re-suspended in a final volume of 1 ml of PBS for further use. Stored beads at 4^{o}C were used up to 6 weeks after preparation.

2.2.4.3 CEC extraction from peripheral blood

CEC were isolated according to an international consensus protocol (Woywodt *et al.*, 2006a). One ml of venous blood was collected into EDTA tubes. Samples were processed within four hours of collection as it has been previously demonstrated that CEC numbers reduce significantly 24 hours after blood collection (Woywodt *et al.*, 2006a). One ml of the collected blood was mixed with 1 ml of PBAC in a 5 ml tube. Twenty µl of Fc receptor–blocking reagent (Miltenyi Biotec) was added and incubated for 5 minutes at room temperature. Fifty µl of the prepared anti-CD146–coated immunomagnetic beads (clone S-endo-1; BioCytex and Dynal Biotech) were then added, and the sample was incubated in the cold room (4°C) for 30 minutes with rotation. Bead-bound cells were separated using a magnet (MPC-L; Dynal

Biotech) and washed 3 times with buffer. Cells were then re-suspended in 90 µl of buffer and 10 µl of a 2-mg/ml preparation of FITC-labeled Ulex europaeus lectin (Sigma-Aldrich) were also added. The sample was then covered with aluminium foil and incubated at room temperature for 1 hour. Cells were finally washed three more times and re-suspended in a final volume of 200 µl buffer.

2.2.4.4 Enumeration of CEC

For the enumeration of CEC, a Nageotte chamber was used on a fluorescence microscope. CEC were defined as cells >10 µm in diameter, Ulex bright, with 5 magnetic beads attached (Woywodt *et al.*, 2006a) (**Figure 2.1**). Nageotte counting chamber holds 50 µl of sample over 40 lines. CEC were counted over the 40 lines and multiplied by 4 to get the total volume per ml of whole blood.

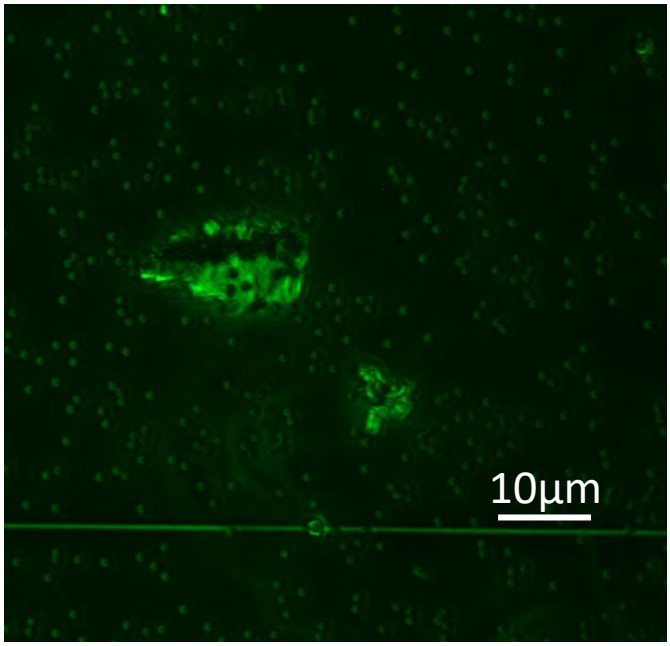

Figure 2.1 Circulating endothelial cell (CEC) identified with fluorescent microscope. *CEC were defined as Ulex bright cells, more than 10µm in size with more than five beads attached.*

2.2.5 Quantification of chemokines/cytokines and soluble molecules

Serum aliquots previously stored at -80^{o}C were used. High-sensitivity C-reactive protein (hs-CRP), serum amyloid A (SAA), angiopoietin 1 and 2, soluble E-selectin, soluble intercellular adhesion molecule 1 and 3 (sICAM-1 and sICAM-3), soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble P-selectin, thrombomodulin (TM), interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), interleukin 10 (IL-10), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 1 beta (IL-1B), tumour necrosis factor-α (TNF-α), interferon-alpha-2a (IFN-α2a), interferon beta (IFN-β), interferon gamma (IFN-γ) and interferon-lambda (IFN-λ) were assessed using a multi-array detection system based on electrochemiluminescence technology (SECTOR Imager 2400; MesoScale Discovery, MSD).

Briefly, multi-array plates pre-coated with capture antibodies were used. Thirty-five µl of each sample was added along with a solution containing detection antibodies conjugated with electrochemiluminescent labels. Analytes in the samples bind to capture antibodies immobilized on the working electrode surface and recruitment of the detection antibodies by the bound analytes completed the sandwich. A buffer was then added which provided the appropriate chemical environment for electrochemiluminescence. The analytes of interest were captured on the relevant electrode. Plates were then loaded into an MSD instrument where a voltage applied to electrodes on each plate caused the captured labels to emit light. The intensity of the emitted light was then measured by the instrument providing a quantitative measurement of analytes in the samples. An important advantage of this system is the ability to simultaneously measure different biomarkers in small (25ul - 50uL) serum or plasma samples. Steps were followed as per manufacturer's protocol.

2.2.5.1 Quantification of Galectin-9

Previously stored serum samples were used from quantification of Galectin-9. The method used was a solid-phase ELISA [solid-phase Human Galectin-9 Quantikine ELISA Kit (R&D systems)]. Briefly, this assay employs the quantitative sandwich enzyme immunoassay technique. Microplates pre-coated with a monoclonal antibody specific for human Galectin-9 were used. 100µl of standards and samples were pipetted into the wells. Present Galectin-9 was bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Galectin-9 was added to the wells followed by further washing to remove any unbound antibody-reagent. The substrate

solution was then added to the wells. The above steps described were followed as per manufacturer's instructions.

2.2.5.2 Quantification of Tumour Necrosis Factor Receptor II (TNFRII)

For the quantification of TNFRII the MSD Human TNF-RII Ultra-Sensitive Kit was used. This is a commercially available detection system based on electrochemiluminescence technology (SECTOR Imager 2400; MSD). The technique followed was the same as described at section 2.2.5 with the exception that 5µl of samples were added to each well.

2.2.6 MP Isolation and enumeration

Stored PPP was thawed quickly in a 37°C water bath and 100-200 µl of plasma were then centrifuged at 16,000g for 1 hour at 4°C. The supernatant was carefully decanted and the resultant MP pellet (usually invisible) was then resuspended in 100µl of 1x Annexin V binding buffer (BD PharMingen, Oxford, United Kingdom). Forty µl of the reconstituted MP were plated onto the wells of a 96 well U-bottomed plate to be ready for staining with Annexin V and other monoclonal antibodies.

2.2.6.1 MP labelling

For the detection and quantification of MP the following steps were followed: 1µl of each antibody along with 3µl of 1x Annexin V binding buffer (a total volume of 10µL) was added to

the 40µl of each sample in order to achieve a 50x final dilution of each antibody. The following antibodies were used (**Table 2.1**):

- Annexin V conjugated with fluorescein brilliant violet 421 (BV421, Biolegend) for binding with phosphatidylserine that is present in all MP.
- R-phycoerythrin (PE) Mouse Anti-Human CD62e, Clone 68-5H11 (Biolegend) for defining endothelial derived MP.
- Brilliant Violet 711 (BV711) Mouse Anti-Human CD19, Clone HIB19 (BD OptiBuild™) for identifying B-cell derived MP.
- Brilliant Violet 605 (BV605) Mouse Anti-Human CD14, Clone M5E2 (Biolegend) for identifying monocyte derived MP.
- Allophycocyanin (APC) Mouse Anti-Human CD3, Clone UCHT1 (Biolegend) for identifying T-cell derived MP.
- To assess tissue factor (TF) expression on monocyte derived MP, samples were stained with mouse fluorescein isothiocyanate (FITC) –labeled anti-human TF, Clone VD8 (American Diagnostica, USA).
- Finally, to exclude a platelet origin of these MP all samples were additionally stained with Peridinin-chlorophyll-protein Complex Conjugate (PerCP) mouse anti-human CD42a, Clone Beb1 (BD Pharmingen).

The samples were then covered with a plate sealer and aluminium foil and placed on an orbital shaker at 500rpm at room temperature for 20min. A total of 150µL of 1x Annexin V binding buffer was added to each sample to terminate the incubation and all samples were

transferred to FACS tubes before being analysed by flow cytometry. Two additional samples were also prepared to serve as negative controls. For the negative control tube, 200 µL of Annexin V Binding Buffer were added to one tube without staining with any antibodies.

Probe	Microparticle type	Fluorochrome Conjugate	Isotype	Clone	Dilution
Annexin V	All	BV421	N/A (Ca^{+2} free)		1:50
CD62e	Endothelial (activation)	PE	IgG2a, κ	HCD62E	1:50
CD14	Monocyte	BV605	IgG2a, κ	M5E2	1:50
CD19	B-cell	BV711	IgG_1, κ	HIB19	1:50
CD42a	Platelet	PerCP	IgG1	Beb1	1:50
CD3	T-cell	APC	IgG1, κ	UCHT1	1:50
CD142	Tissue factor	FITC	IgG1	VD8	1:50

Table 2.1. List of antibodies used for defining different microparticle (MP) populations with flow cytometry.

2.2.6.2 Gating Strategy

MP were simulated using different sizes of standard latex microbeads (0.3 µm, 1.1 µm and 3 µm, Sigma), and a MP gate was determined using these standards. The MP gate included 1.1 µm beads in its upper and outer corner so that it would contain all MP with a size of < 1.1 µm while for the lower limits the 0.3 µm beads were used (**Figure 2.2**). Staining with Annexin V (AnV) was further used to distinguish MP from background electrical noise. Therefore, in this study, MP were defined as events AnV+ and with a size < 1.1µ.

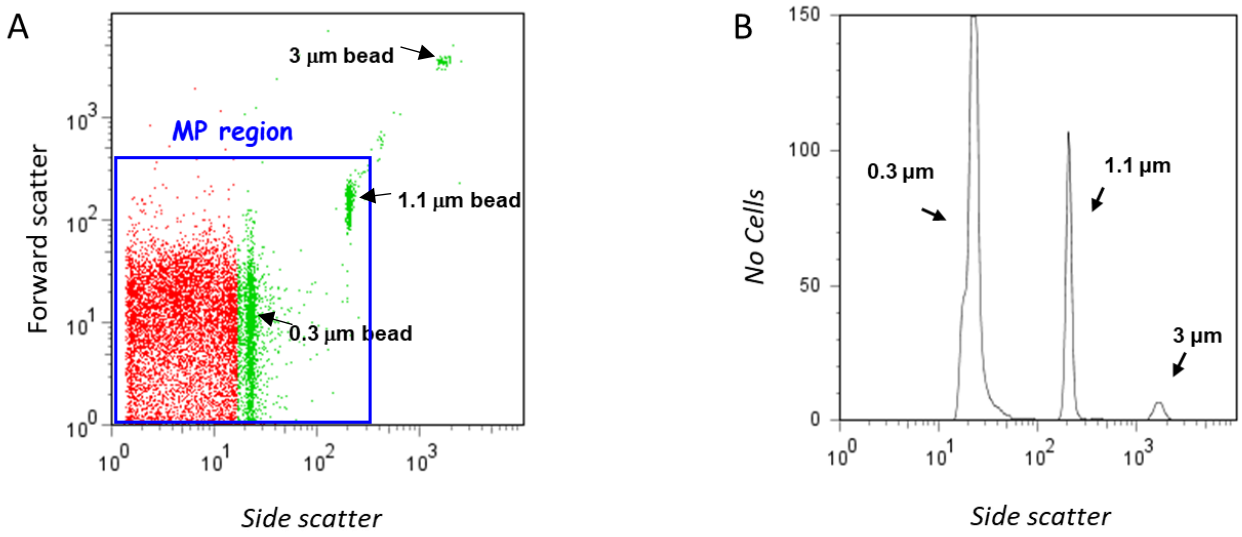

Figure 2.2 (A, B) Gating strategy for microparticles (MP) identification using size-calibrated fluorescent beads ranging from 0.3 to 3 µm*. Standard latex beads, size of 1.1 µm were used to set the gating of MP < 1.1 µm. The 3 µm beads were run concurrently to enable determination of absolute number of MP/ml of plasma (see section 2.2.6.4).*

2.2.6.3 Flow Cytometry analysis

All samples were analysed on a LSRII flow cytometer (BD, UK) with the FACSDiva software (BD Biosciences, NJ, USA). Forward scatter scale, side scatter scale, and each fluorescent channel were set in logarithmic scale. The flow rate was set on low, and all samples were run for 30 seconds. Specific gating for each antibody was achieved by using appropriate isotype controls. As there was no isotype control for Annexin V, BV421 Mouse Anti-Human CD73 was used to set-up with the gating for Annexin V. In addition, the Fluorescence Minus One (FMO) strategy was used, where in separate experiments, the sample of interest was stained for every marker except the one that is being gated. This confirmed adequate compensation and showed any spread of signal from other fluorophores into the channel of interest. The resulting isotype control populations were used to define the positive and negative gates for that marker. MP populations were then defined as particles <1.1µm in size staining for:

a) AnV+/CD62e+/CD42a- were defined as endothelial MP,

b) AnV+/CD3+/CD42a- as T-cell derived MP,

c) AnV+/CD19+/CD42a- as B-cell derived MP, and

d) AnV+/CD14+/TF+ as monocyte derived MP expressing TF (**Figure 2.3**).

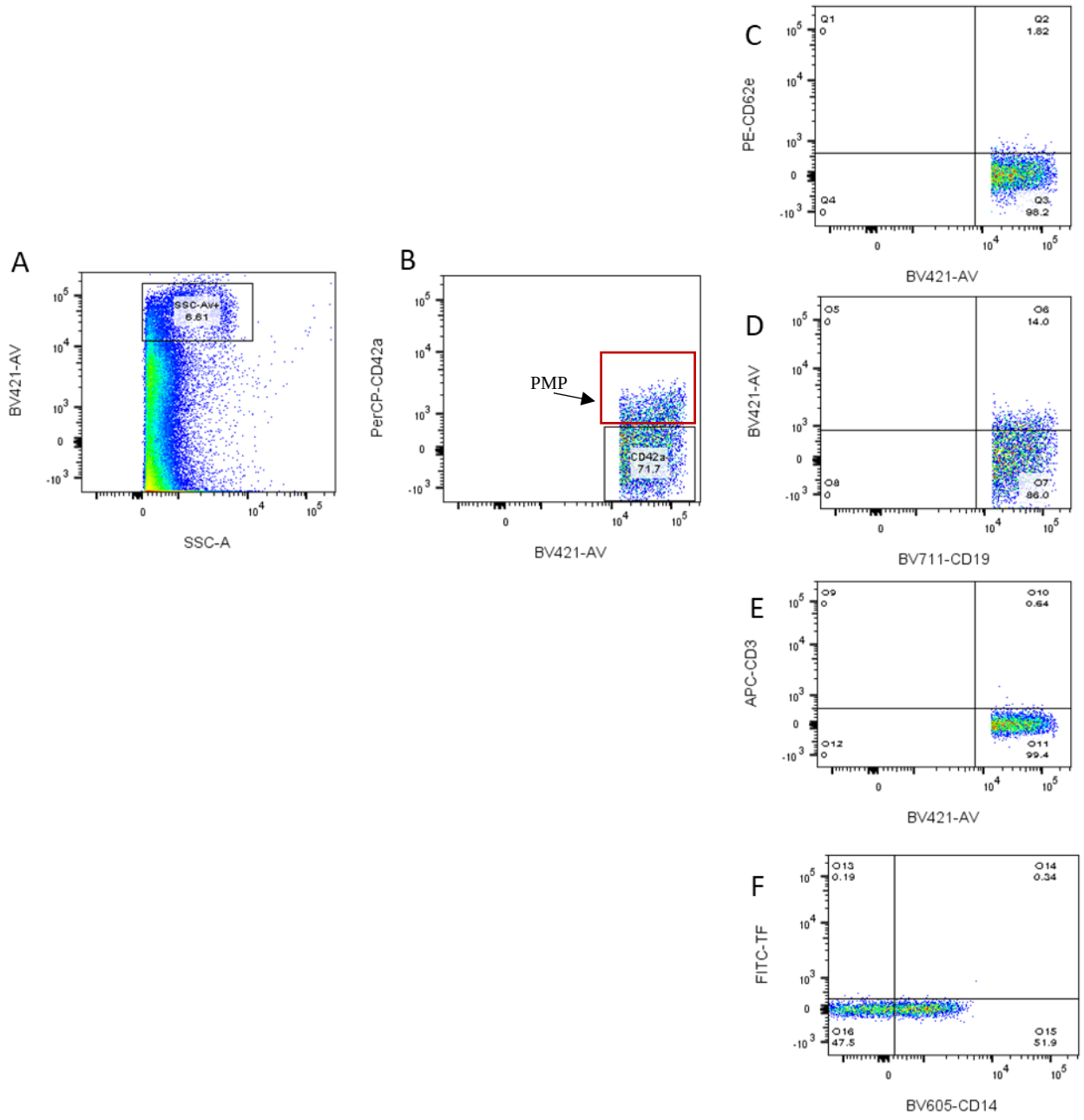

Figure 2.3 Flow cytometry analysis on LSRII Flow Cytometer for detection of circulating microparticles (MP). *The MP population was defined as particles < 1.1 um in size and positive for AnV+.* ***Figure 2.3B*** *Platelet MP (PMP) were defined as AnV+CD42a+particle.* ***Figure 2.3C-F*** *AnV+CD42a- MP population was then used to further characterise endothelial derived MP*

(AnV+CD42a-CD62e+ MP, ***Figure 2.3C****), B-cell derived MP (AnV+CD42a-CD19+ MP,* ***Figure 2.3D****), T-cell derived MP (AnV+CD42a-CD3+ MP,* ***Figure 2.3E****) and tissue factor positive monocytes derived MP (AnV+CD42a-CD14+TF+ MP,* ***Figure 2.3F****), respectively.*

2.2.6.4 MP enumeration

In order to enumerate the MP, 3µm diameter latex beads (Sigma, UK) were run at the same time during every analysis (a total of 200,000 beads was used each time as per manufacturer recommendations). Based on the proportion of beads counted and the total volume of plasma used to obtain the MP, the absolute number of MP per ml of plasma was calculated using the formula below as previously described (Brogan *et al.*, 2004):

$$\text{Absolute number of microparticles/ml of plasma} = \frac{[(200000/\text{no of beads counted}) \times (\text{no of MP counted per well}) \times (\text{no of wells/per sample})]}{\text{mls of plasma used}}$$

Multiplication of the percentage of each MP subpopulation (i.e. endothelial, T-cell etc) with the absolute number of total MP per ml of plasma was then used to get the absolute number per ml of plasma of each one of the MP subpopulation.

2.2.7 Thrombin generation assay

Stored PPP was thawed in a 37°C water bath. Forty µl of PPP were then added to a well of a black polypropylene 96-well plate. Fifty µL of a calcium fluorogenic thrombin substrate (0.5 mM of Z-GG-R-AMC and 7.5 mM of calcium final reagent concentrations, Pathway

Diagnostics) was added and the reaction allowed to proceed for 90min (**Figure 2.4**). All samples were run in duplicate. The plate was read on an Optima fluorescence plate reader (BMG) with excitation/emission spectra of 360/460nm at 1min intervals for 90min and compared to a standard thrombin calibrator (Pathway Diagnostics). No exogenous TF or phospholipids were added, to ensure thrombin generation was mainly due to MP activity. Corn trypsin inhibitor (CTI) was not added, as it has been suggested that the use of CTI could mask the contribution from MP-associated molecules that activate Factor XII (Kluft and Meijer, 2010). Parameters measured were peak thrombin (nM), time to onset of thrombin generation or lag time (min), rate of thrombin generation or velocity index (nM/min), and endogenous thrombin potential (ETP), equivalent to the area under the curve of the thrombogram. A representative thrombogram is shown in **Figure 2.4**.

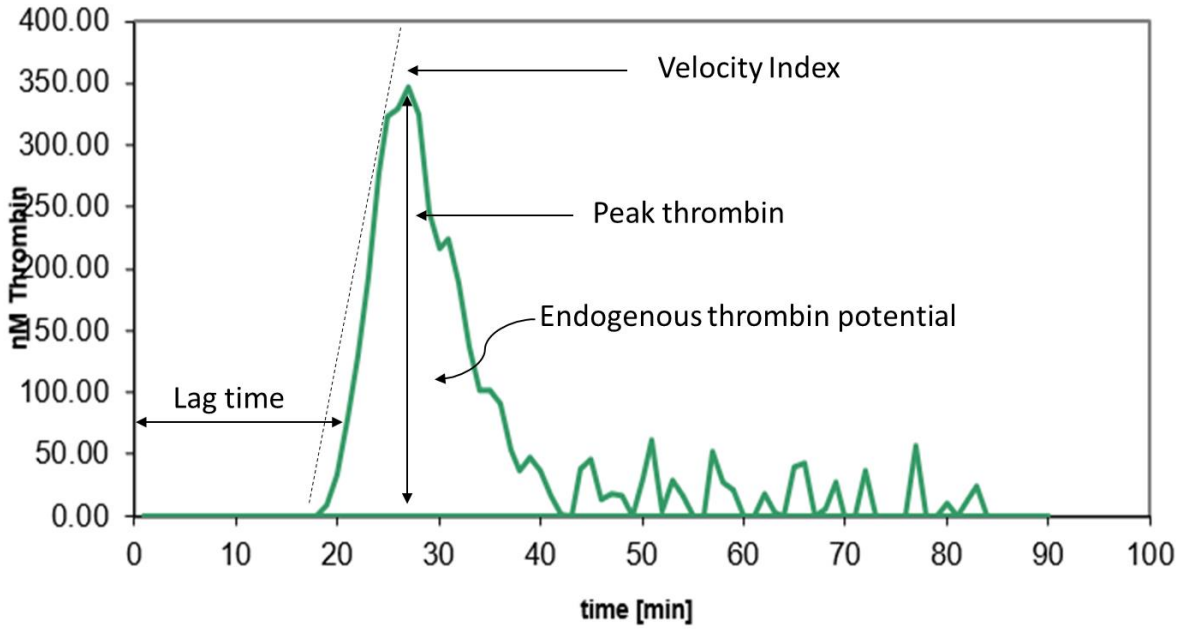

Figure 2.4 An example of a thrombogram generated with the thrombin generation assay (TGA). *A fluorogenic thrombin substrate was added in 40µl of platelet poor plasma to trace thrombin activity over time. The recorded parameters include: a) lag time (min) which corresponds to the time that thrombin starts to generate; b) velocity index which is defined by [peak thrombin/(time to peak thrombin-lag time)]; c) peak thrombin in nM and d) endogenous thrombin potential which corresponds to the area under the curve. Black arrows show lag time, peak thrombin and endogenous thrombin potential, with the gradient of the dashed line showing velocity index.*

2.2.8 Statistical analysis

A number of parametric and non-parametric statistical analyses were used. Biomarker analyses first compared patients with active JDM, inactive JDM, and healthy controls using Kruskal-Wallis test for non-normally distributed data. Other group comparisons were performed using a Mann Whitney U test. Results were expressed as median and interquartile range (IQR). The Chi-square test was used to examine categorical variables. Spearman's correlation was used to explore the relationships between study parameters. The Wilcoxon matched pairs signed rank test was used to compare variables at initial presentation and at latest follow-up for those monitored prospectively. Receiver operator characteristic (ROC) curves were calculated to examine the diagnostic characteristics of indices described. Sensitivity, specificity and likelihood ratios were then calculated to examine the potential diagnostic test characteristics of variables for monitoring disease activity. Youden index (J) was used to estimate the optimal cut-off point that optimizes the biomarker's differentiating

ability. Levels of agreement for CEC counts between different investigators were summarised using Bland–Altman plots. Results are expressed as bias SD and 95% CI of limit of agreement. P values of less than 0.05 (two sided) were regarded as significant. The software Statistica (release 6.0, StatSoft Corporation, Tulsa, OK, USA), Stata (release 7.0) and GraphPad Prism (San Diego, CA) were used for data analyses. Data are presented using GraphPad Prism (San Diego, CA).

3 Circulating endothelial cells in children with Juvenile Dermatomyositis (JDM)

3.1 Summary

Background: Vasculopathy is considered central to the disease pathogenesis of JDM and may underpin disease severity. One major hurdle to the study and detection of the vasculopathy of JDM to date, has been a lack of non-invasive biomarkers. Recently, a number of methods for detecting endothelial cell components in blood which allow non-invasive assessment of vascular injury have been described: one of them is circulating endothelial cells (CEC).

Objectives: To explore the vasculopathy of JDM by assessing biomarkers of endothelial injury (elevated CEC) and investigate their relation to disease activity in children with JDM.

Methods: Ninety patients recruited to the UK JDM Cohort & Biomarker Study were included and studied cross-sectionally. The median age was 10.21 (IQR, 6.68 – 13.40) years with median disease duration of 1.63 (IQR, 0.28-4.66) years at the time of recruitment. A total of 57 (75.0%) were females. Inactive disease was defined as per modified PRINTO criteria: no skin rashes, CK ≤150 U/l, CMAS ≥48/52, MMT8 ≥78/80, PhyGLOVAS ≤0.2/10. Eighty-two healthy control children were recruited. The median age of healthy controls was 16.8 (IQR, 11.8-17.5) years old. CEC were identified with immunomagnetic bead extraction. Results are expressed as median and interquartile range.

Results: CEC were higher in JDM patients at a median of 96 (IQR, 40-192) cells/ml compared to a median of 12 (IQR, 8-24) cells/ml in 82 age-sex matched healthy controls, $p<0.0001$. Patients with active JDM had higher CEC than those with inactive JDM, $p=0.0072$. Patients

with calcinosis had higher CEC compared to healthy controls, $p<0.0001$. In 25 patients studied prospectively, CEC remained stable when there was no change in disease status; there was a decrease in CEC level in patients that went into remission ($p=0.03$), and an increase in CEC level in patients that had a disease flare.

Conclusion: The finding of raised CEC in active JDM emphasises that endothelium is a likely target for autoimmune injury in JDM despite clinical heterogeneity, and could serve as a non-invasive biomarker of vasculopathy of JDM.

3.2 Introduction

Since the introduction of corticosteroids in the 1960s, the mortality of JDM has significantly reduced from about 30% to less than 2% (Huber and Feldman, 2005), but there remains significant variability in long-term outcome. Despite the development of new therapeutic strategies, and significant progress in understanding the underlying disease pathogenesis, a significant proportion of JDM are resistant to treatment, and develop complications. Long-term outcomes likely depend on several factors: genetic, disease severity, organ involvement, and treatment toxicity, (probably) amongst others. Currently, predicting these different disease trajectories remains challenging. Several studies have suggested that the presence and persistence of vasculopathy defines disease severity and relates to poorer long-term outcomes (Spencer-Green et al., 1982; Miles et al., 2007; Gitiaux et al., 2016). Several cohort studies have focused on identifying histological (Miles *et al.*, 2007; Deakin *et al.*, 2016), clinical (Christen-Zaech et al., 2008; Stringer et al., 2008; Challa et al., 2018) and laboratory biomarkers (Stringer et al., 2008; Tansley and McHugh, 2014) that potentially associate with worse disease courses. The lack of non-invasive, easy to use laboratory parameters to detect and monitor the vasculopathy of JDM remains an unmet need. Several endothelial activation markers have been suggested in adults and children (Guzman et al., 1994; Bloom et al., 1995; Kubo et al., 2000; Bloom et al., 2002; Xu et al., 2017). It is important though to highlight that most of the currently studied endothelial activation biomarkers appear to lack specificity as markers used may also be expressed by other cells and do not differentiate activation from endothelial damage and dysfunction (Shantsila et al., 2008). Therefore, CEC may be a better highly specific biomarker for the assessment of endothelial damage and dysfunction.

Endothelial cells form a single layer lining the wall of all blood vessels, and control the interaction between blood components and the surrounding tissues (Minami and Aird, 2005). These cells have several functions: regulation of homeostasis (Galley and Webster, 2004), immune and inflammatory responses (Szekanecz and Koch, 2004; Cook-Mills and Deem, 2005), angiogenesis (Sumpio *et al.*, 2002), and maintenance of an appropriate thrombogenic state (Yau *et al.*, 2015). Endothelial activation, injury, or dysfunction has been shown to be a key player in many pathological conditions: aging, atherosclerosis, inflammatory diseases, and thrombosis (Sumpio *et al.*, 2002). During these pathological responses, the endothelium is activated resulting in the detachment of endothelial cells from the vessel wall and release in the blood circulation. These detached endothelial cells are referred to as CEC (Sabatier *et al.*, 2009). Although the exact mechanism(s) of endothelial cell detachment from the vessel wall is not fully understood, current evidence suggests that endothelial cell detachment can be the result of vascular injury, of cytokines/proteases, or of defective adhesive properties (Wu *et al.*, 2007). In healthy people, CEC are found in very low numbers (usually less than 10/ml whole blood) reflecting normal physiological endothelial turnover, but their number increases substantially in several pathological conditions including autoimmune, inflammatory and cardiovascular diseases (Blann *et al.*, 2005). CEC therefore represent necrotic or activated endothelial cells released into the peripheral circulation following detachment from the vessel wall (Woywodt *et al.*, 2003b).

CEC were first described by Bouvier and Hladovec in 1970 (Gaynor *et al.*, 1970; Hladovec *et al.*, 1978). From 1970 till the early 1990s, several studies identified these cells in various conditions based on their morphological criteria (Hladovec *et al.*, 1976; Sinzinger *et al.*, 1988) raising a lot of questions about the reliability and specificity of the techniques used to detect them (Jaffe, 1987; Percivalle *et al.*, 1993; Erdbruegger *et al.*, 2006). In 1991, an endothelial cell specific monoclonal antibody, S-Endo1, recognizing the CD146 molecule which plays a significant role in cell-cell interaction and signalling (Bardin *et al.*, 2001), became available. In 2001, another monoclonal antibody against CD146, P1H12, was identified (Solovey *et al.*, 2001) and used for detection of CEC in patient with sickle cell anaemia. The availability of CD146 detection established immunomagnetic isolation as a standard methodology for CEC identification and enumeration (Woywodt *et al.*, 2003b; Woywodt *et al.*, 2006a), as described in more detail in section 2.2.4. The use of another endothelial specific stain, Ulex Europaeus lectin-1 (UEA-1), along with an Fc blocking agent to block non-specific binding to leukocytes, led to increased technique specificity (Woywodt *et al.*, 2006a). According to an international consensus definition, CEC are identified as cells with > 10μ diameter, and at least 5 anti-CD146-coated immunobeads attached to their membrane (Woywodt *et al.*, 2006a).

Flow cytometry represents an alternative technique for CEC isolation (Mancuso *et al.*, 2003b; Del Papa *et al.*, 2004; Holmen *et al.*, 2005). Briefly, CEC are detected and differentiated from other cell types using different fluorochrome-conjugated antibodies. Multicolour flow cytometry is quick and allows multi-parametric analyses, but also has several limitations due to the various antibodies and cell surface markers used, cell viability and staining, and gating

strategies employed that make it almost impossible to compare results between different investigators and studies (Khan *et al.*, 2005; Clarke *et al.*, 2008; Danova *et al.*, 2016). It is characteristic that CEC numbers differ substantially between different flow cytometry studies (Mancuso *et al.*, 2003a; Del Papa *et al.*, 2004), suggesting a possible error in gating strategies and flow parameters which is specifically relevant in analysis of rare events (Blann *et al.*, 2005; Khan *et al.*, 2005). Some of the problems identified include the use of lysis buffer that may prevent recovery of CEC (Clarke *et al.*, 2008), the small blood volume used for the detection of rare events (Lanuti *et al.*, 2012), the different surface markers deployed in different studies, sampling and storage methods used (Zhou *et al.*, 2018). Recently, Zhou *et al*. published an optimised five-colour flow cytometry method where all the above parameters were examined, with promising results (Zhou *et al.*, 2018).

To date, CEC have been studied in a number of different conditions (Mutin *et al.*, 1999; Wang *et al.*, 2005) and their role has been investigated by several specialties including rheumatology, nephrology, cardiology, and oncology. CEC have been extensively studied in patients diagnosed with anti-neutrophil cytoplasmic antibody (ANCA)-associated small vessel vasculitis, and are elevated in patients with active vasculitis when compared with patients in remission (Woywodt *et al.*, 2003b; Haubitz and Woywodt, 2004). Moreover, Clarke *et al*. demonstrated that CEC numbers in children with primary systemic vasculitis are associated with disease activity, and can be used for monitoring treatment responses (Clarke *et al.*, 2010). Likewise, in patients with SLE, CEC were found to be markedly elevated in patients with active disease (Clancy, 2000). Similar findings have been shown in other connective

tissue diseases (Del Papa *et al.*, 2004). Yu *et al*. demonstrated that CEC numbers correlate with the severity of coronary artery injury in patients with Kawasaki Disease (Yu *et al.*, 2004). Shah *et al*. recently demonstrated that CEC remained elevated years after the diagnosis of Kawasaki disease in those with coronary artery aneurysms (CAA), even after remodelling of CAA (Shah *et al.*, 2015).

In conclusion, CEC are elevated in a number of diseases associated with endothelial injury and damage. Therefore, CEC might serve as a non-invasive biomarker to detect endothelial injury associated with the vasculopathy of JDM.

3.3 Aims

The aims of this study were to investigate:

(i) whether children with JDM have increased number of CEC compared to healthy controls;

(ii) whether CEC differ between patients with active versus inactive JDM.

3.4 Methods

3.4.1 Patient population and study design

In the cross-sectional part of this study, children with JDM presenting to GOSH from September 2015 to December 2017 were recruited. Children and their families were approached during their inpatient stay (newly diagnosed) or their outpatient clinic follow-up

appointment. Definitions and inclusion/exclusion criteria were discussed in the previous sections (2.1.1 and 2.1.4). Healthy controls were recruited as described in section 2.1.2. Patients were also evaluated prospectively at time of recruitment and their latest follow-up visit.

3.5 Results

3.5.1 Inter-observer variability

I first wanted to establish whether there was any variability in CEC measurements as assessed by different researchers. Bland-Altman plots of CEC counts for 8 consecutive patients with JDM as counted by an experienced post-doctoral research fellow (Dr Ying Hong), my supervisor (Dr Despina Eleftheriou) and myself are summarized in **Figure 3.1**. Bland-Altman analysis was performed to determine the inter-observer variability for CEC enumeration, showing a bias of 0.4286 with SD 5.028 and 95% CI of agreement from -9.427 to 10.28. These results suggested good reproducibility of results amongst different observers.

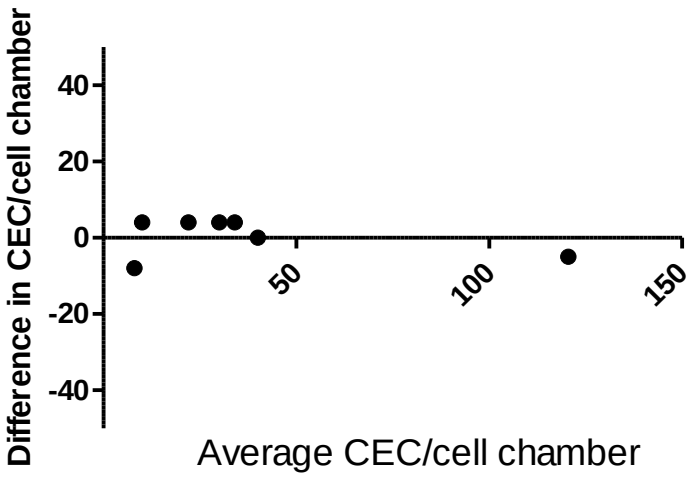

Figure 3.1. Circulating endothelial cell (CEC) enumeration- inter-observer variability.

Bland-Altman plot of CEC counts for 8 consecutive patients exploring the agreement between two investigators for enumeration of CEC. Analysis shows a bias of 0.4286 with SD 5.028 and 95% CI of agreement -9.427 to 10.28.

3.5.2 Demographic, clinical and laboratory data

A total of 90 children with JDM were identified during the study period with a median age of 10.21 (IQR, 6.68–13.40) years old. The median disease duration was 1.63 (IQR, 0.28-4.66) years at the time of recruitment. A total of 57 (75.0%) were females. Sixty-four/90 (71.4%) of the patients had active disease as per modified PRINTO criteria. Eighty-two healthy control children were initially recruited. One subject was excluded from the final analysis as she was suffering from severe eczema and was on systemic steroid treatment at time of assessment thus meeting the exclusion criteria. The median age of healthy controls was 16.8 (IQR, 11.8-17.5) years old. The demographics of the study population alongside routine clinical

laboratory parameters and disease activity measurements are summarised in **Table 3.1** and **Table 3.2**.

Demographics, laboratory parameters and disease activity measures (n JDM patients had testing, if data not available for all 90 JDM patients)	All JDM patients (n = 90)	Patients with active JDM (n = 64)	Patients with inactive JDM (n =26)	P-value[&]
Gender: female (%)	57 (63.3)	48 (75.0)	9 (34.6)	**0.0003**
Disease duration, median (IQR), years	1.63 (0.28-4.66)	0.87 (0.02-3.99)	4.14 (1.80-7.03)	**0.0005**[§]
Age, median (IQR), years	10.21 (6.68-13.40)	10.21 (6.00-14.04)	10.56 (6.94-12.14)	0.9894[§]
Age at disease onset, median (IQR), years	5.48 (3.40-9.25)	5.72 (3.80-9.99)	4.44 (2.72-6.76)	**0.0339**[§]
Time to diagnosis, median (IQR), years	0.34 (0.17-0.69)	0.34 (0.17-0.79)	0.30 (0.17-0.50)	0.8215[§]
Patients with positive antinuclear antibodies	57 (63.3)	43 (67.2)	14 (53.8)	0.2339
Patients with positive Myositis Specific Antibodies (n=49)	33 (67.3)	27 (64.3)	6 (85.7)	0.2630**
SRP	4 (12.1)	4 (14.8)	0 (0.0)	
NXP2	10 (30.3)	7 (25.9)	3 (50.0)	

TIF1g	8 (24.2)	8 (29.6)	0 (0.0)	
MDA5	4 (12.1)	3 (11.1)	1 (16.7)	
JDM Patients that had a muscle biopsy at time of diagnosis	46 (51.1)	37 (57.8)	9 (34.6)	**0.046**
Histopathology Visual Analogue Scale (1.0–10.0 cm), median (IQR)	5.0 (2.0-7.0)	5.0 (3.0-7.0)	3.0 (2.0-7.0)	0.2799§
Haemoglobin, median (IQR), g/L	124 (116-131)	121 (113-127)	127 (120-137)	**0.0025§**
Leukocytes x 10^9/L, median (IQR)	6.47 (5.30-7.90)	6.56 (5.21-8.43)	6.40 (5.51-7.21)	0.8353§
Neutrophils x 10^9/L, median (IQR)	3.51 (2.58-4.46)	3.61 (2.59-4.54)	3.47 (2.52-4.16)	0.6743§
Lymphocytes x 10^9/L, median (IQR)	1.94 (1.32-2.55)	1.85 (1.30-2.59)	2.31 (1.61-2.55)	0.1729§
Platelets x 10^9/L, median (IQR)	310 (247-356)	307 (245-355)	321 (255-373)	0.7535§
Erythrocyte sedimentation rate, median (IQR), mm/h (n = 89)	9 (4-18)	10 (5-20)	4 (3-14)	**0.0330§**
C-reactive protein, median (IQR), mg/dl (n = 89)	5 (5-5)	5 (5-5)	5 (5-6)	0.6314§

Creatinine Kinase, median (IQR), U/L (n=89)	89 (69-138)	84 (66-220)	93 (78-122)	0.7821[§]
Lactate Dehydrogenase, median (IQR), U/L (n=87)	651 (560-809)	694 (583-829)	581 (540-653)	**0.0008[§]**
Alanine aminotransferase, median (IQR), U/L (n=88)	26 (15-42)	29 (17-54)	23 (11-30)	0.0550[§]
Manual Muscle Testing-8, median (IQR) (n=88)	78 (67-80)	74 (59-80)	80 (78-80)	**0.0001[§]**
Childhood Myositis Assessment Score, median (IQR) (n=89)	50 (44-52)	48 (37-52)	52 (50-52)	**0.0005[§]**
Childhood Health Assessment Questionnaire, median (IQR) (n=79)	0.125 (0.000-0.625)	0.25 (0.000-1.000)	0.000 (0.000-0.125)	**0.0043[§]**
Physician Global Assessment, median (IQR) (n=89)	1.1 (0.2-2.7)	2.0 (0.70-3.10)	0.2 (0.0-0.2)	**<0.0001[§]**
Parent/Patient Global Assessment, median (IQR) (n=81)	0.7 (0.0-4.0)	1.9 (0.0-5.0)	0.0 (0.0-0.60)	**0.0015[§]**
Pain Global Assessment, median (IQR) (n=79)	0.2 (0.0-1.8)	0.9 (0.0-3.0)	0.0 (0.0-0.2)	**0.0090[§]**
Treatment at time of recruitment	56 (62.2)	40 (62.5)	16 (61.5)	0.93
Prednisolone	28 (31.1)	24 (37.5)	4 (15.4)	**0.04**

Methotrexate	41 (45.6)	28 (43.7)	13 (50.0)	0.59
Intravenous Immunoglobulin (IVIG)	4 (4.4)	3 (4.7)	1 (3.8)	1.00**
Cyclophosphamide	1 (1.1)	1 (1.6)	0 (0.0)	N/A
Rituximab	1 (1.1)	1 (1.6)	0 (0.0)	N/A
Tumour necrosis factor-alpha inhibitors	10 (11.1)	6 (9.4)	4 (15.4)	0.47**
Other	5 (5.6)	4 (6.2)	1 (3.8)	N/A
*Data are number (%), unless otherwise indicated. [&]P values refer to chi-square test unless otherwise indicated; **Fisher exact test, [§]P: Mann Whitney U test. JDM = Juvenile dermatomyositis; IQR = interquartile range; MDA5 = Melanoma Differentiation-Associated protein 5; NXP2 = Nuclear Matrix Protein 2; SRP = Signal Recognition Pattern. Erythrocyte sedimentation rate: reference range < 10 mm/h, C-reactive protein: reference range < 20 mg/l, creatine kinase: reference range 6–330 U/l, alanine aminotransferase: reference range 10-35 U/l, lactate dehydrogenase: reference range 450–770 U/l.				

Table 3.1. Demographics, laboratory parameters and disease activity measures for 90 patients with Juvenile dermatomyositis (JDM) studied cross-sectionally.

Clinical features and disease activity measures at the time of diagnosis	JDM patients (n = 90)
Proximal muscle weakness	85 (94.4)
Myalgia	67 (74.4)
Arthralgia	45 (50.0)
Arthritis	27 (30.0)
Joint contractures	24 (26.7)
Calcification	8 (8.9)
Skin ulceration	18 (20.0)
Active rash	84 (93.3)
Dysphagia (n=89)	29 (32.2)
Dysphonia	28 (31.1)
Interstitial Lung Disease	18 (20.0)
Cardiac involvement	3 (3.3)
Nailfold capillary changes	74 (82.2)
Fever	22 (24.4)
Haemoglobin, median (IQR), g/L (n=89)	117 (109-125)
Leukocytes x 10^9/L, median (IQR) (n=89)	6.85 (5.61-9.61)
Neutrophils x 10^9/L, median (IQR) (n=89)	3.34 (2.57-5.24)
Lymphocytes x 10^9/L, median (IQR) (n=89)	2.23 (1.44-3.08)

Platelets x 10^9/L, median (IQR) (n=89)	300 (240-365)
Erythrocyte sedimentation rate, median (IQR), mm/h (n = 89)	12 (8-25)
C-reactive protein, median (IQR), mg/dl (n = 81)	5 (5-7)
Creatine Kinase, median (IQR), U/L (n=89)	290 (102-2000)
Lactate Dehydrogenase, median (IQR), U/L (n=88)	1173 (820-1847)
Manual Muscle Test-8, median (IQR) (n=84)	52 (38-64)
Childhood Myositis Assessment Scale, median (IQR) (n=83)	30 (16-41)
Physician Global Assessment, median (IQR) (n=70)	4.5 (2.6-6.6)
Parent/Patient Global Assessment, median (IQR) (n=70)	4.0 (1.7-6.0)
Pain Global Assessment, median (IQR) (n=65)	3.0 (1.0-5.5)
Histopathology score at vascular domain in muscle biopsy (n=46)	1.0 (0.0-2.0)
Total JDM muscle biopsy score (n=46)	11 (6-17)
*Data are number (%), unless otherwise indicated. IQR = interquartile range; JDM = Juvenile dermatomyositis	

***Table 3.2**. Clinical features for 90 patients with Juvenile dermatomyositis (JDM) studied cross-sectionally as assessed at time of initial diagnosis.*

Overall the patient series had characteristics similar to those of previously published UK JDM cohorts (McCann *et al.*, 2006). The median age of disease onset was 5.48 (IQR, 3.40-9.25) years with a median time from disease onset to diagnosis 0.34 (IQR, 017-0.69) years. Positive MSA were found in 33/49 (67.3%) of tested patients with NXP2 being the predominant type. Children with active disease at the time of recruitment had higher ESR (p=0.0330) and LDH (p=0.0008) when compared with children with inactive disease, while there was no difference with regards to CK and CRP levels.

3.5.3 Circulating endothelial cells levels are elevated in JDM

CEC were higher in JDM patients at a median of 96 cells/ml (IQR, 40-192) compared to a median of 12 cells/ml (IQR, 8-24) in 81 age-sex matched healthy controls, p<0.0001 (**Figure 3.2**). Patients with active JDM had higher CEC than those with inactive JDM, p<0.0001. Patients with calcinosis had higher CEC compared to healthy controls, p<0.0001 (**Figure 3.3**). CEC did not significantly differ between JDM patients with inactive disease when they compared according to whether they were on treatment at time of assessment or not, p=0.53 (**Figure 3.4**).

Further analysis of specific disease features showed the following: CEC were found to be higher in patients who had nailfold capillaries changes present with CEC counts at a median of 128 cells/ml (IQR, 72-248) compared to patients with normal nailfold capillaries who had CEC of a median of 48 cells/ml (IQR, 32-119), p=0.0007 (**Figure 3.5**).

Patients who had positive TIF1g antibodies had higher number of CEC at a median of 200 cells/ml (IQR, 128-452) when compared with patients with NXP2 positive antibodies at a median of 36 cells/ml (IQR, 15-56), p=0.0005, respectively (**Figure 3.6**).

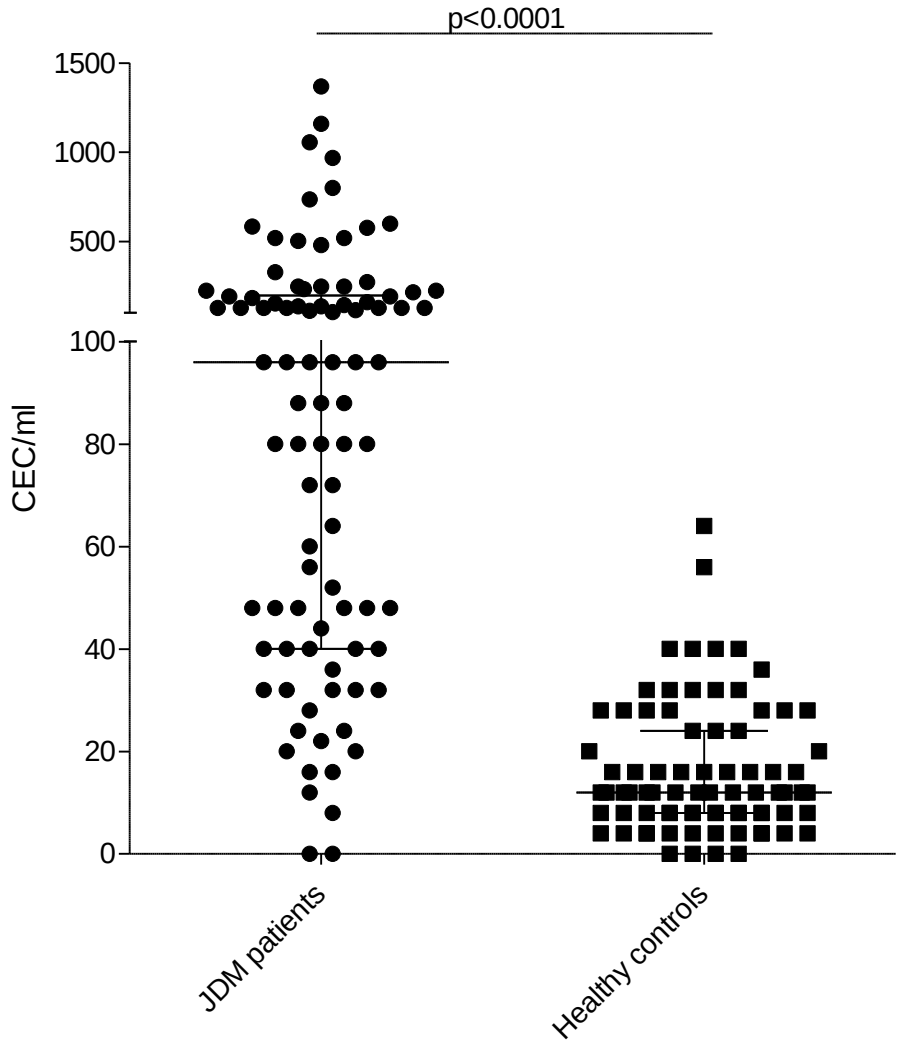

Figure 3.2. Circulating endothelial cells (CEC) in Juvenile dermatomyositis (JDM) patients compared to healthy controls. *Patients with JDM had significantly higher level of CEC when*

compared to healthy controls (p<0.0001). P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.

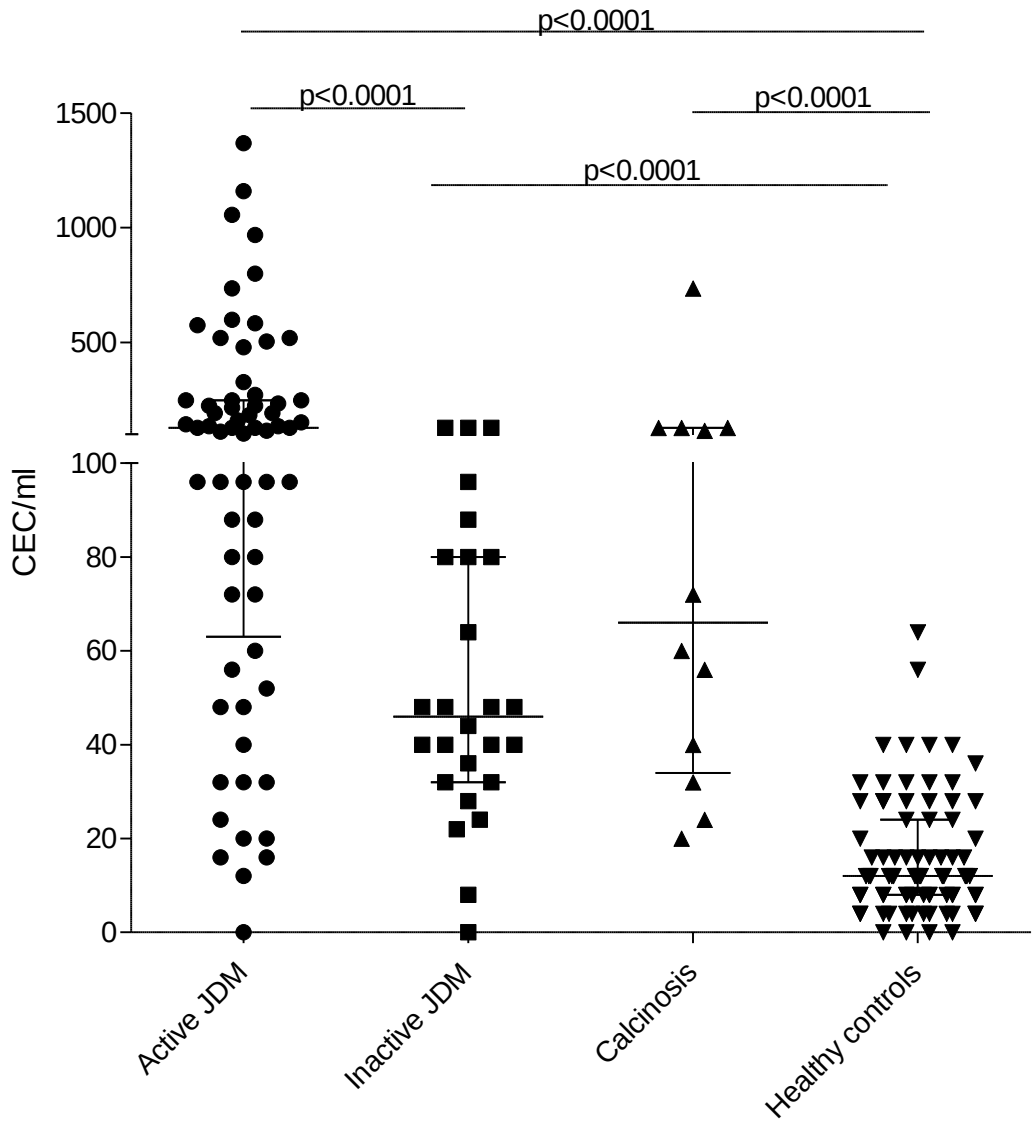

Figure 3.3. Circulating endothelial cell (CEC) levels in Juvenile dermatomyositis (JDM) patients with active and inactive disease and those with calcinosis. *Children with active JDM had higher number of CEC at a median of 128 cells/ml (interquartile range, IQR, 66-248) when compared to patient with clinically inactive disease with a median of 46 cells/ml (IQR, 32-80),*

p<0.0001 and healthy controls, p<0.0001. Moreover, patients with inactive disease had higher numbers of CEC when compared to healthy controls, p<0.0001. Finally, patients with calcinosis had higher CEC median 66 cells/ml (IQR, 34-128) compared to healthy controls at a median of 12 cells/ml (IQR, 8-24), p<0.0001. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.

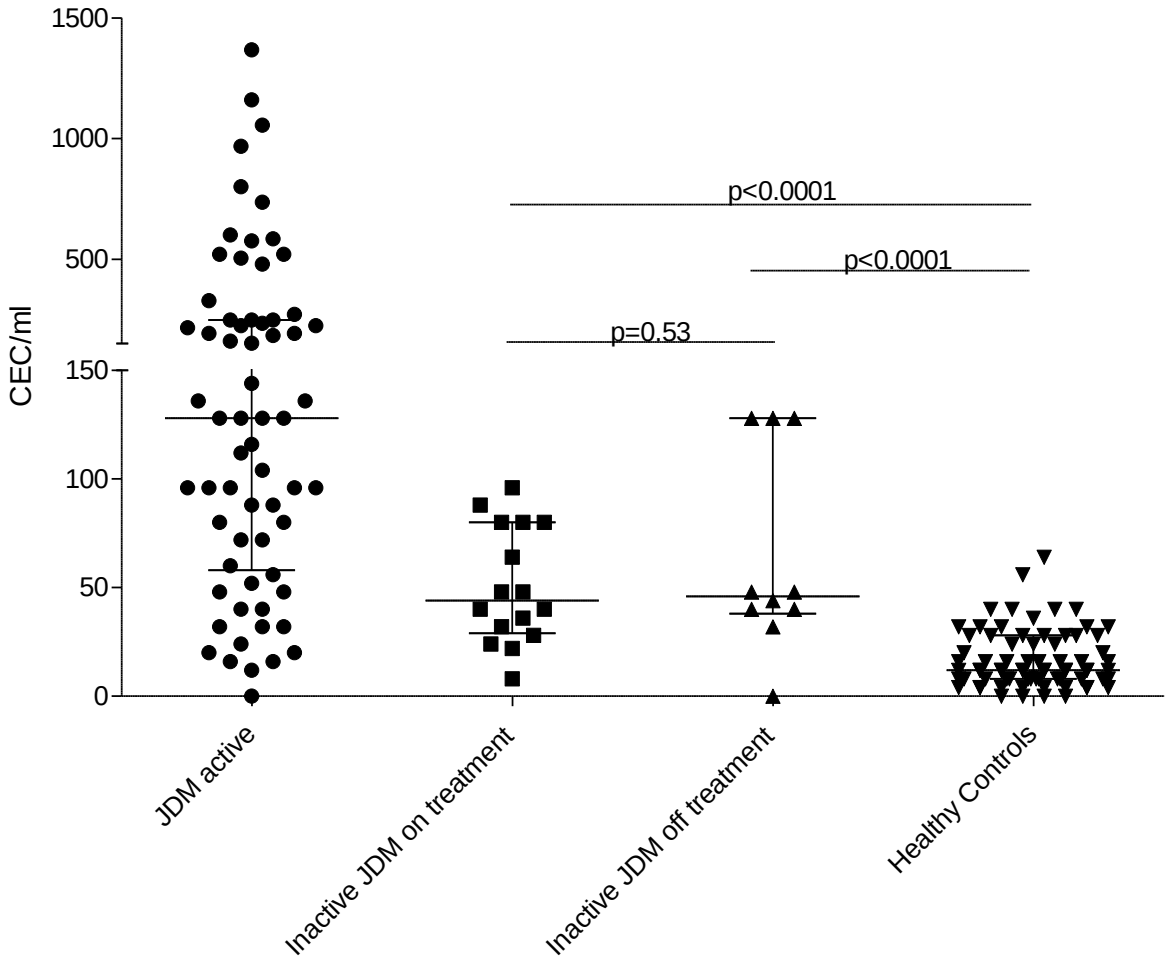

Figure 3.4. Circulating Endothelial Cells (CEC) in patients with inactive Juvenile dermatomyositis (JDM) that were either on or off treatment compared to healthy controls. *Numbers of CEC did not differ significantly between patients with inactive disease on*

treatment when compared to those off treatment, p=0.53. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.

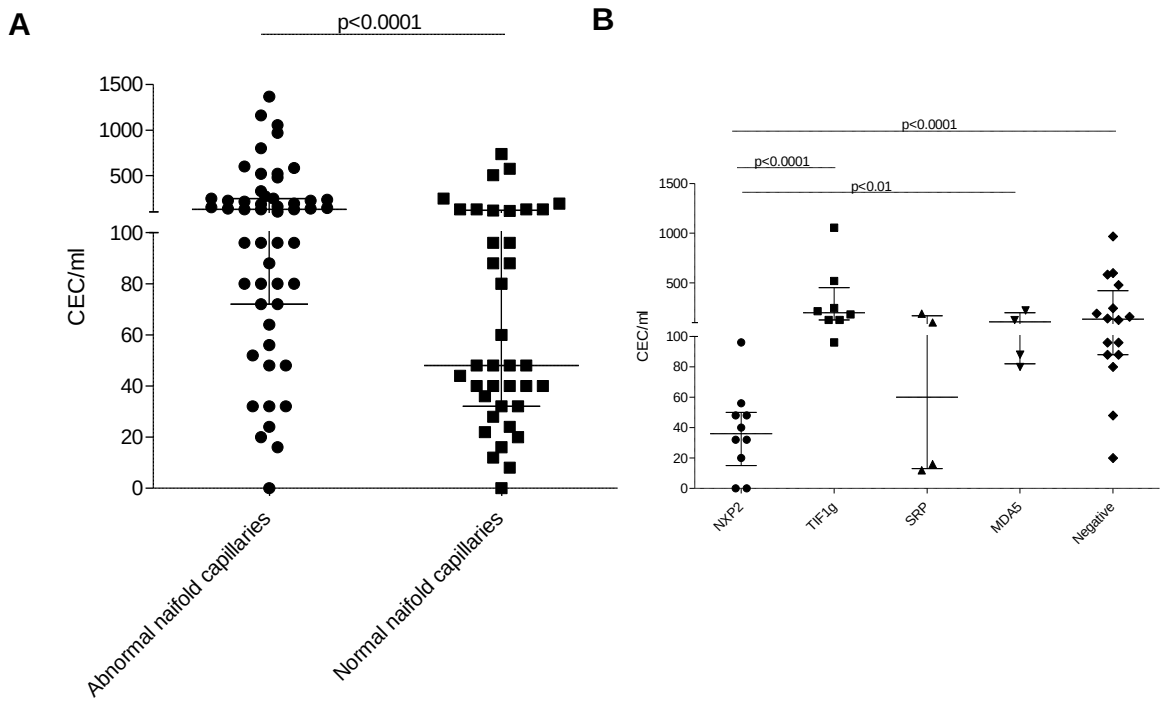

Figure 3.5. Circulating endothelial cells (CEC) levels in Juvenile dermatomyositis (JDM) patients based on the presence of abnormal nailfold capillaries (Figure 3.5A) and myositis specific antibody (MSA) subgroup (Figure 3.5B). *Patients with abnormal nailfold capillaries had higher CEC: median 128 cells/ml (interquartile range, IQR, 72-248) compared to patients with normal nailfold capillaries with a median 48 cells/ml (IQR, 32-119), p=0.0007.* ***B.*** *Patients with Transcription Intermediary Factor 1 (TIF1g) positive antibodies had higher CEC median 200 cells/ml (IQR, 128-452) compared to patients with positive Nuclear Matrix Protein -2 (NXP2) antibodies with a median 36 cells/ml (IQR, 15-56), p=0.0005. Myositis Specific Antibody (MSA) data were available in 49 patients with JDM. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

When considering muscle biopsy scores, patients scoring ≥ 1 it the vascular domain had numerically higher (but non-statistically significant) numbers of CEC when compared with patients with score 0 (**Figure 3.6**).

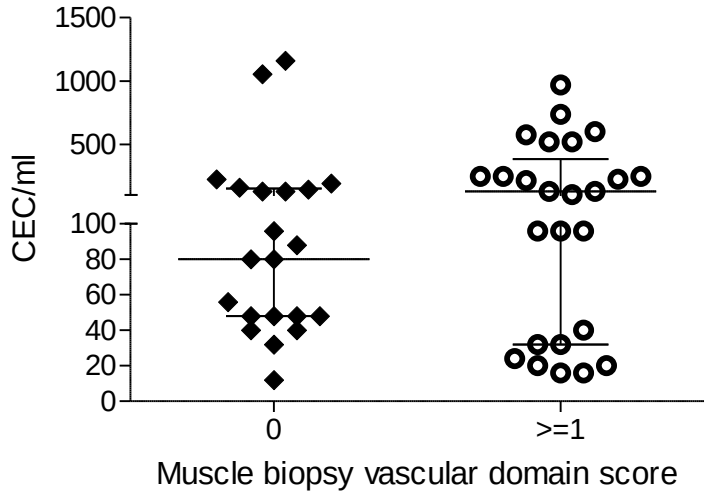

Figure 3.6. Circulating endothelial cells (CEC) for Juvenile dermatomyositis (JDM) patients with muscle biopsy scores available (n=46). *Patients with higher scores in the muscle biopsy vascular domain (≥ 1) had slightly higher CEC median 128 cells/ml (interquartile range, IQR, 32-384) compared to patients with scoring of 0 who had a median of 80 cells/ml (IQR, 48-152) but this did not reach statistical significance, p=0.45. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

3.5.4 Longitudinal changes of CEC in children with JDM

In addition to the cross-sectional study, 25 children, with a median age of 11.22 (IQR, 8.16-14.05) years were studied prospectively at time 0 (time of recruitment), and during at least

one follow-up visit. Seventeen patients had active disease at recruitment and 8 were in remission. Three of the patients with initially clinically inactive disease had a disease flare during the study follow-up period. In all three, there was a flare of skin disease with no evidence of muscle involvement. During the second study visit, eleven patients had ongoing disease activity while another eleven were in remission.

In the prospective part of the study, there was no significant difference in the number of CEC at time 0 (time of recruitment) and at last follow-up visit, p=0.37. CEC remained stable when there was no change in disease status; but there was a decrease in CEC level in patients that went into remission (p=0.03), and an increase in CEC level in patients that had a flare of the disease (**Figure 3.7**).

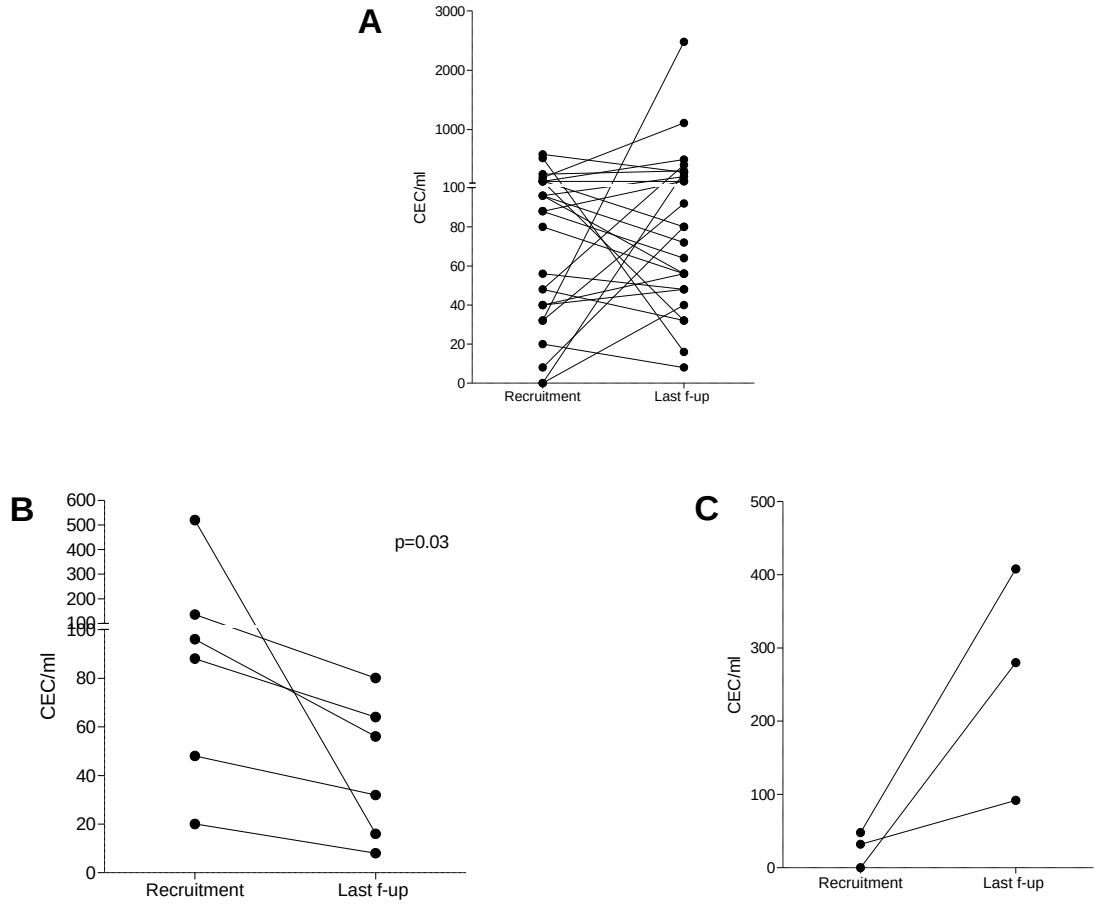

Figure 3.7. Prospective study of circulating endothelial cells (CEC) in children with Juvenile dermatomyositis (JDM). *A. CEC in patients studied prospectively at time of recruitment and last follow-up visit. B. There was a significant decrease in CEC levels (p=0.03) in patients with active disease at time of recruitment that were in remission at last follow-up visit and C. An increase in the CEC levels in patients in remission at the time of recruitment that had a flare of their disease at last follow-up visit. P value was calculated with Wilcoxon matched pairs signed rank test.*

3.5.5 Test characteristics of CEC for identification of children with active JDM

On the basis of the above analysis demonstrating that CEC could serve as a biomarker of disease activity, the test characteristics of CEC for identification of active JDM disease by plotting ROC curves were then examined. The ROC curve for CEC at varying definitions of positivity is shown in **Figure 3.8**. The full test characteristics of CEC for definition of active disease are summarized in **Table 3.3**.

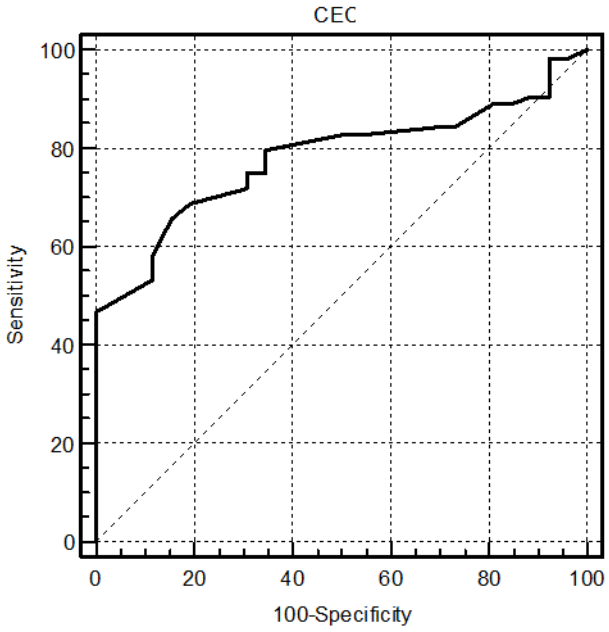

Figure 3.8. Receiver operator characteristic (ROC) curve for circulating endothelial cells (CEC) as a biomarker for Juvenile dermatomyositis (JDM) activity.

Definitions of test positivity were considered at varying levels of CEC counts as shown here. Best cut-off as calculated by the Youden Index was defined as CEC/ml > 88 with sensitivity 65.6% and specificity 84.6%, a positive likelihood of 4.27 and a negative likelihood of 0.41.

Area under the ROC curve = 0.782, Standard error = 0.049, 95% Confidence interval = 0.682 to 0.862.

Criterion	Sensitivity (95% C.I.)	Specificity (95% C.I.)	+LR	-LR
>=0	100.0 (100.0-100.0)	0.0 (0.0- 0.0)	1	
> 0	98.4 (91.6-99.7)	3.8 (0.6-19.7)	1.02	0.41
> 8	98.4 (91.6-99.7)	7.7 (1.2-25.2)	1.07	0.2
> 12	96.9 (89.1-99.5)	7.7 (1.2-25.2)	1.05	0.41
> 16	93.8 (84.7-98.2)	7.7 (1.2-25.2)	1.02	0.81
> 20	90.6 (80.7-96.5)	7.7 (1.2-25.2)	0.98	1.22
> 22	90.6 (80.7-96.5)	11.5 (2.6-30.2)	1.02	0.81
> 24	89.1 (78.7-95.5)	15.4 (4.5-34.9)	1.05	0.71
> 28	89.1 (78.7-95.5)	19.2 (6.6-39.4)	1.1	0.57
> 32	84.4 (73.1-92.2)	26.9 (11.6-47.8)	1.15	0.58
> 36	84.4 (73.1-92.2)	30.8 (14.4-51.8)	1.22	0.51
> 40	82.8 (71.3-91.1)	46.2 (26.6-66.6)	1.54	0.37
> 44	82.8 (71.3-91.1)	50.0 (29.9-70.1)	1.66	0.34
> 48	79.7 (67.8-88.7)	65.4 (44.3-82.8)	2.3	0.31
> 52	78.1 (66.0-87.5)	65.4 (44.3-82.8)	2.26	0.33
> 56	76.6 (64.3-86.2)	65.4 (44.3-82.8)	2.21	0.36
> 60	75.0 (62.6-85.0)	65.4 (44.3-82.8)	2.17	0.38

> 64	75.0 (62.6-85.0)	69.2 (48.2-85.6)	2.44	0.36
> 72	71.9 (59.2-82.4)	69.2 (48.2-85.6)	2.34	0.41
> 80	68.7 (55.9-79.8)	80.8 (60.6-93.4)	3.58	0.39
> 88 *	65.6 (52.7-77.0)	84.6 (65.1-95.5)	4.27	0.41
> 96	57.8 (44.8-70.1)	88.5 (69.8-97.4)	5.01	0.48
> 104	56.2 (43.3-68.6)	88.5 (69.8-97.4)	4.87	0.49
> 112	54.7 (41.7-67.2)	88.5 (69.8-97.4)	4.74	0.51
> 116	53.1 (40.2-65.7)	88.5 (69.8-97.4)	4.6	0.53
> 128	46.9 (34.3-59.8)	100.0 (100.0-100.0)		0.53
> 136	43.7 (31.4-56.7)	100.0 (100.0-100.0)		0.56
> 144	42.2 (29.9-55.2)	100.0 (100.0-100.0)		0.58
> 152	40.6 (28.5-53.6)	100.0 (100.0-100.0)		0.59
> 160	39.1 (27.1-52.1)	100.0 (100.0-100.0)		0.61
> 184	37.5 (25.7-50.5)	100.0 (100.0-100.0)		0.62
> 192	34.4 (23.0-47.3)	100.0 (100.0-100.0)		0.66
> 216	32.8 (21.6-45.7)	100.0 (100.0-100.0)		0.67
> 224	29.7 (18.9-42.4)	100.0 (100.0-100.0)		0.7
> 234	28.1 (17.6-40.8)	100.0 (100.0-100.0)		0.72
> 248	23.4 (13.8-35.7)	100.0 (100.0-100.0)		0.77
> 272	21.9 (12.5-34.0)	100.0 (100.0-100.0)		0.78

> 328	20.3 (11.3-32.2)	100.0 (100.0-100.0)		0.8
> 480	18.8 (10.1-30.5)	100.0 (100.0-100.0)		0.81
> 504	17.2 (8.9-28.7)	100.0 (100.0-100.0)		0.83
> 520	14.1 (6.7-25.0)	100.0 (100.0-100.0)		0.86
> 576	12.5 (5.6-23.2)	100.0 (100.0-100.0)		0.88
> 584	10.9 (4.5-21.3)	100.0 (100.0-100.0)		0.89
> 600	9.4 (3.5-19.3)	100.0 (100.0-100.0)		0.91
> 736	7.8 (2.6-17.3)	100.0 (100.0-100.0)		0.92
> 800	6.2 (1.8-15.3)	100.0 (100.0-100.0)		0.94
> 968	4.7 (1.0-13.1)	100.0 (100.0-100.0)		0.95
> 1056	3.1 (0.5-10.9)	100.0 (100.0-100.0)		0.97
> 1160	1.6 (0.3-8.4)	100.0 (100.0-100.0)		0.98
> 1368	0.0 (0.0-0.0)	100.0 (100.0-100.0)		1

Table 3.3. ***Test characteristics of circulating endothelial cells (CEC) for definition of Juvenile dermatomyositis (JDM) activity.*** *LR+ = Likelihood ratio for a positive test result; LR-=Likelihood ratio for a negative test result; CI=confidence intervals.*

3.5.6 Correlation of CEC with common laboratory data and muscle weakness and general function assessment scores

As CEC were found to differ significantly between patients with active disease and patients with clinically inactive disease, I then decided to explore the relationship between CEC and common laboratory parameters and other routinely used in clinical practice disease activity assessment scores. CEC did not correlate with CK but there was a significant but weak correlation with LDH (p=0.03) (**Figure 3.9**). When the scores of disease activity tools were assessed, there was no correlation between CEC and CMAS but there was an association between CEC and MMT-8 (p=0.04) and PhyGLOVAS (p=0.0002), respectively (**Figure 3.10**).

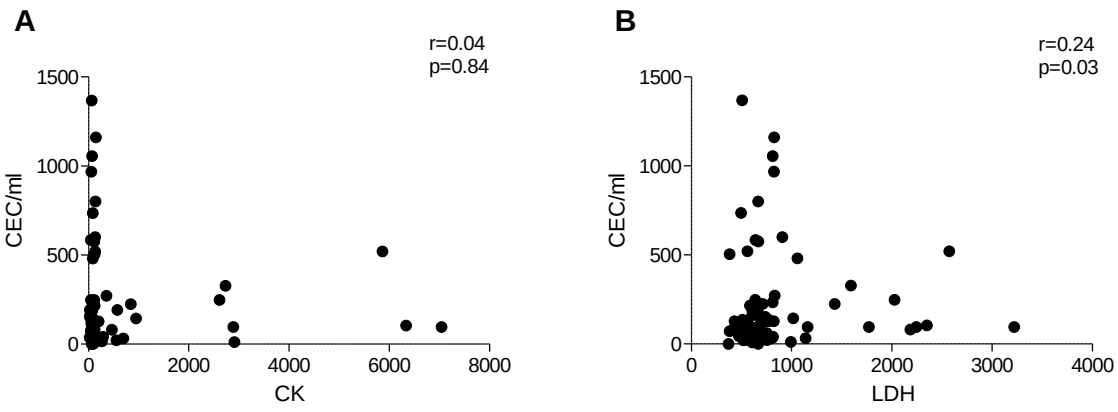

Figure 3.9. Correlation of Circulating Endothelial Cells (CEC) level with Creatinine Kinase (CK) (A) and Lactose Dehydrogenase (LDH) (B). *There was no correlation between CEC and CK, p=0.84) while Juvenile dermatomyositis (JDM) patients with higher levels of LDH demonstrated also higher numbers of CEC, p=0.03. P values were calculated using Spearman's correlation test.*

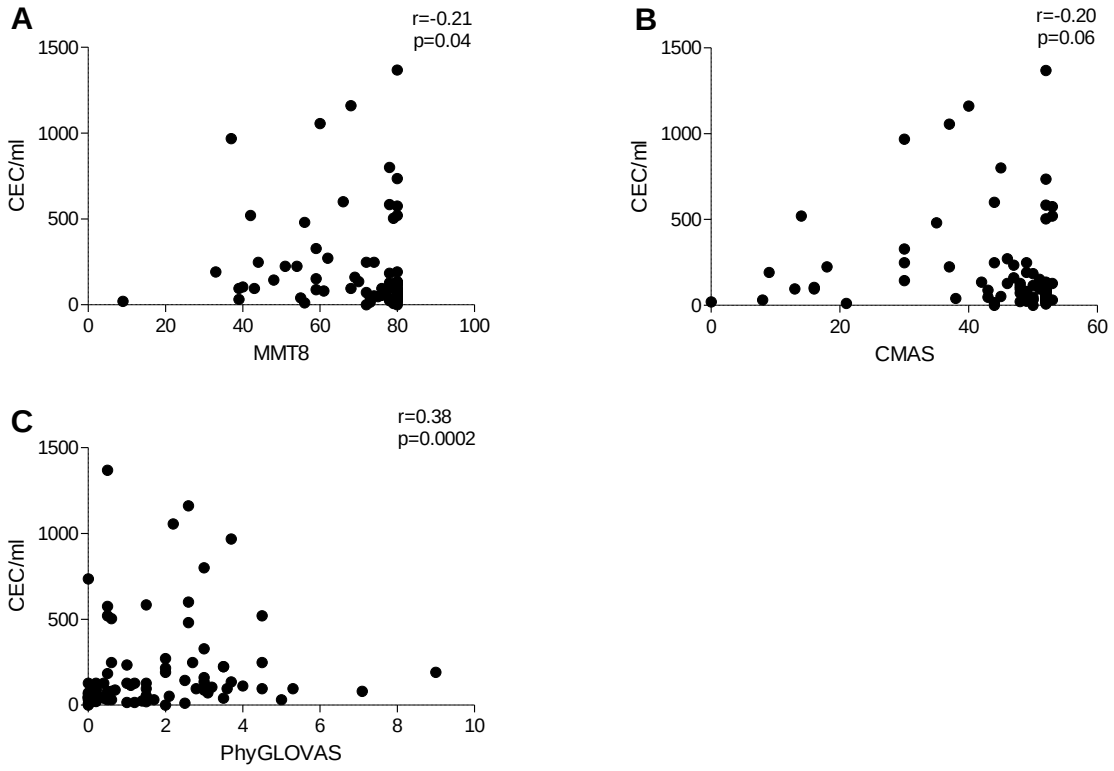

Figure 3.10. Correlation of Circulating Endothelial Cells (CEC) with scores of disease activity tools using non-parametric spearman's correlation test. *CEC number did not correlate with Childhood Myositis Assessment Score (CMAS, p=0.06), but associations were demonstrated with Manual Muscle Testing -8 (MMT-8, p=0.04) and Physician Global Assessment in Visual Analogue Score (PhyGLOVAS, p=0.0002) scores.*

3.6 Discussion

In this cross-sectional study of a group of children with JDM, CEC were identified as a possible biomarker that tracked endothelial injury and differed significantly between patients with active disease, patients with clinically inactive disease and healthy child controls. When a

small number of patients were studied prospectively, CEC levels remained stable in the patients with the same disease status at the two studied time points, but differed significantly in patients with active disease at recruitment who went into remission when subsequently studied. Thus, CEC might be a useful non-invasive marker that can both detect and monitor vascular injury over time in children with JDM, and might have prognostic significance, although this is currently unknown.

JDM represents the commonest idiopathic inflammatory myopathy of childhood (Meyer *et al.*, 2015). When looking at affected tissues, vascular and perivascular inflammation is a predominant feature of the disease, thus early descriptions recognise JDM as a systemic angiopathy of childhood (Whitaker and Engel, 1972). The presence and persistence of vascular involvement is associated with more severe and refractory disease and the development of life-threatening complications. With recent therapeutic strategies, survival and outcomes of JDM have improved significantly with a reported mortality of <2% (Martin *et al.*, 2012), but the long-term outcome differs substantially between patients. A significant variable predictive of adverse disease courses is the development of vascular complications. Early recognition of vasculopathic features and early aggressive treatment are key to a better outcome. However currently, predicting and monitoring the development of vasculopathy throughout the disease course remains challenging due to the lack of non-invasive biomarkers. In addition, current therapeutic approaches are untargeted and hence often fail to control disease activity.

CEC detached from the affected vascular wall can provide useful information for studying vascular injury. Increased levels of CEC have been observed in several diseases with widespread inflammatory and non-inflammatory vascular damage, such as in patients with sickle cell anaemia (Solovey *et al.*, 1997; Strijbos *et al.*, 2009), cytomegalovirus infection (Grefte *et al.*, 1993), ANCA-associated vasculitis (Woywodt *et al.*, 2003b), SLE (Kluz *et al.*, 2007), Behçet's disease (Kutlay *et al.*, 2008) and acute myocardial infarction (Lee *et al.*, 2005). There is also evidence to suggest that control of the underlying disease with appropriate treatment may restore endothelial function and decrease the CEC levels. In systemic autoimmune diseases, in particular, endothelial cells are targets of antibody or immune complex-mediated injury. Previous studies in patients with SLE have demonstrated that patients with active disease had higher numbers of CEC in peripheral blood suggestive of ongoing vascular damage even in patients with no evidence of vasculitic symptoms (Kluz *et al.*, 2007). Moreover, in patients with vasculitis, a paradigm of an endothelial disorder, CEC were found to be a good biomarker of disease activity and thus a useful tool at the hands of treating physicians for monitoring disease course and predicting relapses (Camoin-Jau *et al.*, 2000; Nakatani *et al.*, 2003; Woywodt *et al.*, 2003b; Haubitz and Woywodt, 2004; Blann *et al.*, 2005). This study now suggests that CEC levels are elevated in children with active JDM compared to children with inactive disease and healthy controls. CEC could therefore serve as a biomarker to detect ongoing endothelial injury in patients with JDM and identify those at high risk of developing severe complications with a poor outcome.

It is important to highlight that approximately 60% of the patients included in my study were on immunosuppression at the time of recruitment, but they were still demonstrating elevated numbers of CEC. This indicates that CEC remain a good biomarker of endothelial injury irrespective of treatment. Of course, the levels of CEC are expected to decrease with treatment as the disease is getting better controlled but when there is a flare of the disease with increased endothelial injury, vascular injury can be still detected by CEC numbers as demonstrated in the prospective part of the study. This study has also highlighted that CEC were elevated in patients not only with active disease but also in patients that were considered clinically to be in remission suggesting that there may be ongoing endothelial injury in this group of patients despite current therapeutic management. That finding can have several implications. Speculatively, based on these results, it could be argued that currently used treatments do not effectively target all pathways affected in JDM, which may subsequently contribute to long-term disease complications related to the persistence of vasculopathy (Gitiaux *et al.*, 2016). Thus, having a non-invasive biomarker relating to the vascular component of JDM pathology might prove helpful in predicting ongoing subclinical disease activity and poor outcome, identifying cases resistant to current treatment and help clinicians to adopt stratified approaches to the treatment of each patient.

CEC were found to correlate with some of the traditionally used laboratory markers and tools of disease activity such as the LDH, MMT-8 and PhyGLOVAS; but not with CK and CMAS. This could be attributed to the fact that CEC possibly capture events at a very early stage before there is any change in clinical activity or to poor sensitivity of the existent clinical tools of

disease activity to capture the vascular component of the disease. The poor correlation between CEC and some of the clinical disease activity scores highlights that low grumbling disease activity may still contribute to long-term JDM related vasculopathic sequelae and /or cardiovascular sequelae later into the disease course. To date, clinicians had no specific tool to identify and monitor persistent vasculopathy, further illustrating the urgent clinical need for such a biomarker to be used in routine clinical practice. The finding of elevated CEC in those with abnormal nailfold capillaries does lend support for the use of this item as a clinical sign of JDM vasculopathy. A simplistic, therefore, two-step approach could involve routine bedside nailfold capillaroscopy as a primary, quick diagnostic tool for the presence of vasculopathy followed by enumeration of CEC for further quantification of the effect.

CEC were identified and quantified using immunomagnetic bead extraction. A number of endothelial specific antigens have been used so far for the identification of CEC including CD146, CD141, CD106, CD105, CD62e, CD54 and CD31 (Khan *et al.*, 2005; Erdbruegger *et al.*, 2006). In recent years, CD146 is the most routinely used antigen, which is mainly expressed at the endothelial junction where it plays an important role in the control of intercellular coherence, permeability, and signalization (Anfosso *et al.*, 2001; Bardin *et al.*, 2001). However, CD146 is not restricted to the endothelium and is expressed in small amount on megakaryocytes and specific malignant cells (Haubitz and Woywodt, 2004). Thus, it cannot be used in isolation for the identification of CEC, so a second biomarker is needed to increase specificity. This can be achieved with further staining with FITC-labeled Ulex europaeus lectin (Miettinen *et al.*, 1983). To date, there has been no standardization of the methods used to

quantify CEC. Additionally, as CEC are present in very low numbers in healthy people, the method used for the isolation and enumeration of CEC should be a sensitive technique able to capture rare events. Most studies have used immunomagnetic separation or flow cytometry in blood samples for CEC isolation (George *et al.*, 1992; Woywodt *et al.*, 2006a; Ozdogu *et al.*, 2007; Mariucci *et al.*, 2009). The superior method for isolation of CEC is the immunomagnetic extraction that uses magnetic beads coated with the antibody CD146 that specifically binds to the CD146 antigen in the surface of endothelial cells (Woywodt *et al.*, 2006b). Flow cytometry is another attractive method to quantify CEC. It tends to use a combination of different surface antigens such as CD146, CD45, and CD31 to detect the endothelial cells (Jacques *et al.*, 2008). It has though previously shown that flow cytometry has low sensitivity and demonstrates high variability due to its inability to detect low number of cells (Clarke *et al.*, 2008). For example, significant differences have been reported in the levels of CEC in healthy controls ranging from 4 to 1300 cells per ml of peripheral blood, depending on the applied method (Goon *et al.*, 2006; Widemann *et al.*, 2008). To overcome these limitations, Chen *et al*. (Chen *et al.*, 2017) recently described a novel microfluidic assay for the enumeration and identification of CEC. In addition, Sabashnikov *et al*. (Sabashnikov *et al.*, 2017) suggested an optimization of a previously described method characterised by two steps: firstly, the immuno-magnetic pre-enrichment of a CD34+ sub-population of cells from peripheral blood; and secondly, the flow cytometric detection and enumeration (absolute cell number per sample) of CEC using an integrated workflow. If those methods prove to be reliable and reproducible, it would be of great interest to be used also in children with JDM as they may enable further characterisation of CEC in regard to their vascular bed of origin

with the use of multicolour flow cytometry. It would be of great interest and clinical significance to be able to identify the anatomic origin of those cells (i.e. gastrointestinal tract, lung parenchyma, myocardium) and guide further management.

This study has several limitations. JDM is rare and thus the study population was relatively small and heterogeneous; MSA subgroups were even smaller, and so further statistical analyses was not performed. At time of recruitment, patients were on different treatment regimes. Subgroup analysis of treatments was not done due to relatively small numbers. It is important to highlight that ciclosporin, used in several paediatric rheumatology centres for the treatment of JDM, has been previously demonstrated to be associated with increased numbers of CEC which may reflect of endothelial toxicity caused by calcineurin inhibitors. (Woywodt *et al.*, 2003a). Thus, CEC may be found in very high levels in patients treated with ciclosporin despite good clinical response and may represent a meaningless biomarker of disease activity in that patient cohort. Moreover, treatment with ciclosporin itself may contribute to worse long-term outcomes due to the deleterious effects of calcineurin inhibitors on endothelial morphology and function (Lau *et al.*, 1989; Collins *et al.*, 1993; Iurlaro *et al.*, 1998; Morris *et al.*, 2000). All the included patients in this study were from a single study centre (GOSH) (none of them treated with ciclosporin), thus results could differ if patients from other study centres were included. Finally, blood samples were processed in an unblinded way and thus results could be biased. Having said that, comparison of the numbers obtained by me with two blinded researchers did not indicate significant differences in counting CEC.

In summary, three key findings from my study and previous research in this area are:

1) CEC can non-invasively track endothelial injury in children with JDM;

2) CEC were increased not only in patients with active JDM but also in patients with inactive disease suggestive of ongoing endothelial injury despite clinically inactive disease, and

3) CEC correlated only with few traditional tools of disease activity, demonstrating a lack of routine clinical biomarkers able to capture the vasculopathic component of the disease.

Further study is now required to look into the clinical implications of this ongoing endothelial injury. Moreover, identification of the vascular bed origin of CEC would be of great interest as it could help identify and monitor specific organ involvement, predict long-term prognosis and direct clinician's decision about therapeutic management.

4 Assessment of circulating cytokines and other circulating vascular biomarkers in children with JDM

4.1 Summary

Background: Several molecules and proteins are known to be released upon endothelial cell activation/damage which may play a key role into the pathogenesis of JDM vasculopathy, and as potential biomarkers for monitoring disease activity and guiding treatment.

Objectives: To compare inflammatory and endothelial injury/activation protein profiles in patients with JDM and healthy controls, and further examine the relation to disease activity, other biomarkers of endothelial injury, and the presence of severe extra-muscular manifestations related to JDM vasculopathy.

Methods: Ninety patients with JDM were included in this part of study. Patients were classified as having inactive disease as per previously mentioned PRINTO modified criteria. Soluble adhesion molecules and proteins associated with endothelial activation were quantified using a validated commercial multi-array detection system based on electrochemiluminescence technology (SECTOR Imager 2400; MesoScale Discovery); for galectin -9 enzyme-linked immunosorbent assay (ELISA) was used.

Results: Patients with JDM had increased levels of soluble adhesion molecules, suggestive of endothelial activation compared to healthy controls. Enhanced type I IFN signature was also observed in patients with active disease compared to patients with inactive disease, and healthy controls. Galectin-9 level was the strongest predictor of disease activity, and most strongly correlated with levels of CEC ($r=0.48$, $p<0.001$).

Conclusion: Patients with JDM had increased levels of biomarkers of endothelial activation/dysfunction. Galectin-9, mainly expressed by endothelial cells, demonstrated the best diagnostic accuracy in discriminating between patients with active disease and patients with inactive disease in children with JDM.

4.2 Introduction

The endothelium constitutes a complex organ that interacts with its environment and can quickly change between quiescent to highly activated state. The activated endothelium has pro-thrombotic and pro-inflammatory properties necessary for the fight against pathogens and toxins, which can potentially become deleterious if uncontrolled, and for maintenance of haemostasis. It has been previously shown that endothelial activation and dysfunction plays a vital role in the pathogenesis and progression of numerous diseases including viral infections (Page and Liles, 2013), cardiovascular disease (Widlansky *et al.*, 2003), and immune-mediated conditions (Kluz *et al.*, 2007).

4.2.1 Soluble adhesion molecules

Pathological conditions, including inflammatory processes, may disrupt the “sleeping” phase of endothelial cells and endorse trans-endothelial migration of cells and expression of molecules needed at certain sites. The activated leukocytes- endothelial cells interactions are facilitated by selectins, that are signalling molecules consisting of lipids and chemokines, integrins and their ligands, and junctional molecules (Nagaraju *et al.*, 2006). Many of these

molecules have also been shown to play a significant role in endothelial cell activation, proliferation and differentiation. Studies in adults with autoimmune diseases have investigated the function of soluble adhesion molecules. The levels of VCAM-1, E-selectin, ICAM-1 and ICAM-3, which are key players for the adhesion and migration of leukocytes through the endothelium towards inflamed sites, have been investigated in several rheumatological diseases (Mojcik and Shevach, 1997). Only limited studies have explored the role of these molecules in paediatric rheumatic disease (Bloom *et al.*, 1999). Recently, a study in children with various autoimmune diseases, including 4 patients with JDM, measured serum levels of ICAM-1, ICAM-3, VCAM-1, L-selectin, and E-selectin (Bloom *et al.*, 2002) and demonstrated increased levels of ICAM-1, VCAM-1 and L-selectin in JDM when compared to healthy controls. ICAM-1 was also found to be significantly elevated in patients with active disease compared to patients that were in clinical remission. E-selectin is expressed on activated endothelial cells. In combination with P-selectin, E-selectin helps the migration of leukocytes along the endothelial layer to a site of injury or inflammation promoting leukocyte adhesion to activated endothelial cells (Aird, 2007). This process is enabled by the upregulation of ICAM-1 and VCAM-1 which are transmembrane proteins of the immunoglobulin superfamily (Collins *et al.*, 1995).

4.2.2 Thrombomodulin

Activated endothelial cells when activated also acquire pro-thrombotic properties. Thrombomodulin (TM) is present on the surface of the endothelial cell stroma, particularly in the smaller vascular surfaces, where it has an antithrombotic role (Page and Liles, 2013).

Levels of surface TM significantly reduce once endothelial cells are activated probably due to the cleavage and release of the molecule in circulation (Faust *et al.*, 2001). Thus, circulating TM can serve as a marker of endothelial cell activation and dysfunction.

4.2.3 Angiopoietins

Angiopoietin-1 (Ang-1) and -2 (Ang-2) are two other potential biomarkers that can prove useful to the monitoring of the endothelial status. Ang-1 is produced in the perivascular cells and smooth muscle cells surrounding the endothelial cell monolayer (van Meurs *et al.*, 2009); Ang-2 is produced and deposited in endothelial cells and their rapid release is triggered by inflammatory stimuli. Ang-1 and Ang-2 binds antagonistically on the Tie-2 receptor, a vascular tyrosine kinase receptor primarily expressed in endothelial cells. The role of angiopoietins in the inflammatory process have been extensively studied in the context of infectious diseases (Page and Liles, 2013), but not much is known about their role in other inflammatory conditions. Upregulation of Ang-1, Ang-2 and Tie-2 was suggested to be a key element to the development of vasculopathy in adult cases of Systemic Sclerosis (SSc) (Moritz *et al.*, 2017).In adults with RA, Ang-1 was proposed as a marker that could help establish an early diagnosis (Ishikawa *et al.*, 2011).

4.2.4 Interleukin-6

A number of cytokines have been studied in the pathogenesis of DM and JDM but so far no reliable cytokine biomarker has been identified for differentiation between active and inactive disease. IL-6 is a soluble mediator with a wide effect on inflammation, immune

response and haematopoiesis (Tanaka *et al.*, 2014). It is produced by several cells including endothelial cells, monocytes, B and T lymphocytes (Hirano, 1998). In a murine lupus model, it has been demonstrated to be involved in B-cell activation, a significant component for autoimmune disease onset (Finck *et al.*, 1994). IL-6 serum levels were found to be elevated in several autoimmune diseases such as SLE (Ohl and Tenbrock, 2011), SSc (Kitaba *et al.*, 2012) and RA (Ohshima *et al.*, 1998). In children and adults with IIM, serum levels of IL-6 have been found to be increased and to correlate with disease activity (Reed *et al.*, 2012; Yang *et al.*, 2013). In another comparative study, IL-6 levels were much lower in IIM when compared to RA suggesting that IL-6 plays a less significant role in IIM compared to RA (Cronstein, 2007). When looking into specific organ involvement, JDM patients with ILD had higher IL-6 levels compared to those without ILD (Wakiguchi *et al.*, 2015), indicating that IL-6 could serve as a possible biomarker of disease activity and might relate to specific organ involvement.

4.2.5 Interleukin-1β

IL-1β is a multifunctional, highly inflammatory cytokine the production of which is the result of inflammasome activation (Awad *et al.*, 2018). The presence of IL1β+3953T polymorphism has been identified as potential risk factor for JDM in children of Caucasian ancestry (Mamyrova *et al.*, 2008). In addition, studies looking at the profile of cytokines in affected muscles of DM patients have demonstrated increased expression of IL-1α and IL-1β, indicating that IL-1β may play an important role in disease pathogenesis (Lundberg *et al.*, 1997; Nyberg *et al.*, 2000). Although, IL-1β has been found to be increased in the serum of SLE patients,

especially when the disease is associated with renal involvement (Cigni *et al.*, 2014), evidence is sparse in regards to JDM patients.

4.2.6 Interleukin-8

IL-8 has a variety of proinflammatory properties including immune cell activation (Baggiolini *et al.*, 1994) and promotion of angiogenesis (Koch *et al.*, 1992). It is produced by a number of different cells types including endothelial cells and monocytes. Overproduction of IL-8 contributes to the development of chronic inflammatory diseases as demonstrated for RA (Seitz *et al.*, 1991), inflammatory bowel disease (Grimm *et al.*, 1996), psoriasis (Schroder *et al.*, 1992) and small vessel vasculitis (Cockwell *et al.*, 1999). All these diseases are characterised by the presence of activated neutrophils in lesional areas and elevated IL-8 production. In IIM, IL-8 has been found not only to correlate with disease activity (Reed *et al.*, 2012; Gono *et al.*, 2014) but serum levels of IL-8 were higher in anti-MDA5 patients with ILD and served as a poor predictor for fatal outcome in that subgroup of patients (Gono *et al.*, 2014).

4.2.7 Interleukin-10

IL-10 is an anti-inflammatory cytokine produced by B- and T-cells, monocytes and macrophages (Moore *et al.*, 2001). It has several modulatory effects on the immune system including down-regulation of Th1 cytokines and MCH class II, but also stimulation and prolongation of survival of autoreactive B-cells, highlighting its importance in autoimmune diseases (Llorente *et al.*, 1995; Moore *et al.*, 2001). High levels of IL-10 have been documented

in the serum of patients with SSc, Kawasaki disease (Moore *et al.*, 2001) and autoimmune lymphoproliferative syndrome (Lopatin *et al.*, 2001) while in SLE IL-10 also correlates with disease activity (Houssiau *et al.*, 1995). Regarding DM, Aleksza *et al*. did not find any differences in IL-10 levels between DM patient and healthy controls (Aleksza *et al.*, 2005). Similarly, Reed *et al*. failed to demonstrate any association between IL-10 levels and changes in disease activity in a mix cohort of JDM and DM patients, while IL-10 levels were significantly decreased in patients treated with MMF when adjusted for disease activity levels (Reed *et al.*, 2012).

4.2.8 Tumour necrosis factor-α

One of the most investigated inflammatory proteins in dermatomyositis is tumour necrosis factor (TNF)-α which is known to be involved in systemic inflammation (Clark, 2007), and an important therapeutic target in several autoimmune diseases (Radner and Aletaha, 2015; Li *et al.*, 2017; Touhami *et al.*, 2019). Two active forms of TNF- α exist, a membrane-bound and a soluble one, which are produced by numerous immune cells (Doss *et al.*, 2014). TNF induces secretion of IL-1 and IL-6 and it triggers the activation and proliferation of T lymphocytes (Postal and Appenzeller, 2011). In a small study, TNF-α levels were significantly elevated in 15 JDM patients when compared to 5 healthy controls (Xu *et al.*, 2016). TNF-α also moderately correlated with disease activity in other cohorts (Bilgic *et al.*, 2009; Reed *et al.*, 2012). When Gono *et al*. looked into different disease phenotypes, they found that DM patients with ILD had higher levels of TNF-α compared to patients without ILD, which levels significantly decreased after initiation of treatment (Gono *et al.*, 2014). It becomes thus apparent that

serum TNF-α may be a candidate biomarker of disease activity in JDM but larger studies are needed to confirm these preliminary findings.

4.2.9 IFNs and IFN induced chemokines

Recently, researchers have focused their interest on the potential role of IFNs and the IFN induced chemokines both on the pathogenesis of IIM and as biomarkers of disease activity as they are present in the serum and muscle biopsies of children with JDM (Fall *et al.*, 2005; Rice *et al.*, 2017). Although, type I IFNs have been thought to be produced by activated dendritic cells (DC), a recent study by Rodero *et al*. in which IFN-α was measured with a very sensitive assay did not demonstrate increased IFN-α expression either in peripheral DC or other immune cells in JDM patients compared to healthy controls (Rodero *et al.*, 2017). Investigation of the role of IFN-α and IFN-β in JDM is limited by the lack of appropriate assays for the measurement of their levels in the serum. To overcome that obstacle, expression of IFN related genes and IFN related chemokines have been studied. IFN related genes in whole blood of patients with DM and JDM and IFN related chemokines, including monocyte chemoattractant protein-1 (MCP1) and interferon gamma-inducible protein 10 (IP10), were found to be upregulated when compared to healthy controls while they were also found to be related with disease activity measures (Baechler *et al.*, 2007; Bilgic *et al.*, 2009; Reed *et al.*, 2012; Sanner *et al.*, 2014). MCP1 is known to attract and activate T-cells and monocytes while it also known to play an important role in inflammation, angiogenesis and development of atherosclerosis (Yadav *et al.*, 2010), something very interesting in regards to JDM which is a small vessel vasculopathy. IP10 is secreted by several types of cells including endothelial cells

dependent on IFN-γ and is known to have angiostatic properties (Belperio *et al.*, 2000). IP10 has been found to be associated with lung involvement both in SSc (Tiev *et al.*, 2009) and dermatomyositis patients (Gono *et al.*, 2014). Recently, type III IFNs have gained increasing interest in rheumatology with publications mainly focusing in SLE (Amezcua-Guerra *et al.*, 2015), SSc (Dantas *et al.*, 2015) and RA (Wu *et al.*, 2013). Type III IFNs include four different subtypes: IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B) and IFN-λ4. These type III IFNs may play a significant role in autoimmune diseases with studies also suggesting a possible pathogenetic role through enhanced pro-inflammatory cytokine production by monocyte-derived macrophages, dendritic cells and natural killer cells (Megjugorac *et al.*, 2009; de Groen *et al.*, 2015). Moreover, Th17 cells have been demonstrated to produce IFN-λ1 in psoriatic lesions (Wolk *et al.*, 2013). However, the role of type III IFNs has not been explored in IIM.

4.2.10 Galectin-9 and Tumour Necrosis Factor Receptor II

Recently, two more circulating proteins have been suggested as possible biomarkers for monitoring disease activity in patients with JDM: Galectin-9, and Tumour Necrosis Factor Receptor II (TNFRII), each with distinct biological functions (Bellutti Enders *et al.*, 2014). Galectin-9 belongs to a family of lectins that are known to have a central immunomodulatory role in diverse processes such as immune responses, infections, cancer, and atherosclerosis (Balan *et al.*, 2008; Norling *et al.*, 2009). In endothelial cells, galectin-9 can be induced by interferon-γ (IFN-γ) and Toll like receptor 3 activation with a possible role in inflammation (Imaizumi *et al.*, 2002). The exact function of Galectin-9 needs to be further elucidated. In a

small study, galectin-9 was suggested to serve as a good biomarker for monitoring disease activity as galectin-9 levels differed significantly between patients with active disease and those in remission (Bellutti Enders *et al.*, 2014; Enders *et al.*, 2015). Recently, Galectin-9 was proposed as a stable biomarker in the detection of IFN signature in patients with SLE and anti-phospholipid syndrome (van den Hoogen *et al.*, 2018b).

TNF binds to both TNF receptors I and II, and is a key player in TNF mediated diseases (Hehlgans and Pfeffer, 2005). Whereas TNFRI is expressed in most cell types, TNFRII is typically found on immune and endothelial cells (Wajant *et al.*, 2003). Soluble TNFRII is formed from proteolytic cleavage of cell surface TNFRII. The exact mechanism of action of TNFRII is not well known with some studies suggesting a protective role (Teh *et al.*, 2000), and others indicating a probable pathogenic role in autoimmunity (Al-Ansari *et al.*, 2000; Mok *et al.*, 2016). Recently, TNFRII has been suggested as useful biomarker of disease activity in children with JDM (Bellutti Enders *et al.*, 2014).

In summary, studies so far have produced variable results and were often undertaken in limited numbers of patients. In addition, most were in adults not children and no study combined measurements of all these different proteins and markers of endothelial injury or activation.

4.3 Aims

To investigate the inflammatory protein profile associated with endothelial activation and dysfunction in children with JDM, and to examine whether this approach could serve as a biomarker for monitoring disease activity relating to the vasculopathy of JDM.

4.4 Methods

High-sensitivity (hs-CRP), serum amyloid A (SAA), angiopoietin 1 and 2, soluble E-selectin, soluble intercellular adhesion molecule 1 and 3 (sICAM-1 and sICAM-3), soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble P-selectin, thrombomodulin, tumour necrosis factor α (TNF-α), interleukin-1β, 6, 8 and 10, monocyte chemoattractant protein-1 (MCP1), interferons -α, -β, -γ and λ1, interferon gamma-inducible protein 10 (IP10) and Tumour Necrosis Factor Receptor II (TNFRII) were assessed using a multi-array detection system based on electrochemiluminescence technology (SECTOR Imager 2400; MesoScale Discovery) as previously described in chapter 2.2.5 and 2.2.5.2. Galectin-9 was assessed with a solid-phase ELISA [solid-phase Human Galectin-9 Quantikine ELISA Kit (R&D systems)] as previously described in chapter 2.2.5.1.

4.5 Results

The inflammatory protein profile was assessed in 90 patients with JDM. Sixty-four/90 (71.4%) of the patients had active disease as per modified PRINTO criteria. Eighty-two healthy control children were initially recruited. Patients and healthy controls demographic characteristics

have been previously described in section 3.4.1 (**Table 3.1**). Comparisons of two groups are shown in **Table 4.1**.

Cytokines/chemokines and inflammatory molecules	**JDM patients median (IQR) (n patients tested indicated)**	**Healthy Controls median (IQR) (n healthy controls tested indicated)**	**p-value**
hs-CRP, pg/ml	957785 (313130-7.299*10^6) (n=74)	715208 (276079-1.849*10^6) (n=68)	0.3300
SAA, pg/ml	2.280*10^6 (973445-1.002*10^7) (n=74)	2.080*10^6 (1.206*10^6-5.826*10^6) (n=68)	0.9300
Ang-1, pg/ml	59203 (39788-67536) (n=74)	43497 (37171-54133) (n=69)	**0.0113**
Ang-2, pg/ml	4749 (3002-6088) (n=74)	4118 (3629-4889) (n=69)	0.4800
E-selectin, pg/ml	15.53 (11.15-19.38) (n=74)	11.75 (9.13-15.27) (n=68)	**0.0239**
P-selectin, pg/ml	132.6 (111.3-169.8)	100.4 (75.39-134.6)	**0.0044**

	(n=74)	(n-68)	
s-ICAM 1, pg/ml	301621 (233027-374830) (n=74)	231421 (137465-285538) (n=68)	**0.0009**
s-ICAM 3, pg/ml	0.4868 (0.3347-0.7612) (n=74)	0.4629 (0.3700-0.5480) (n=68)	0.3800
s-VCAM 1, pg/ml	459037 (344694-637458) (n=74)	359825 (204596-405884) (n=68)	**0.0018**
TM, pg/ml	4.978 (3.985-6.065) (n=74)	3.713 (3.416-4.326) (n=68)	**0.0017**
TNFRII, pg/ml	6258 (4055-9433) (n=84)	5048 (3748-6354) (n=73)	**0.005**
Galectin-9, ng/ml	76.3 (52.4-134.0) (n=90)	38.4 (27.1-50.8) (n=73)	**<0.0001**
Il-10, pg/ml	0.5 (0.3-0.9) (n=90)	0.3 (0.2-0.5) (n=54)	**<0.0001**
IL-1β, pg/ml	0.09 (0.05-0.13) (n=90)	0.06 (0.02-0.08) (n=54)	**0.003**
IL-6, pg/ml	0.8 (0.3-1.6) (n=90)	0.3 (0.2-0.6) (n=54)	**<0.0001**
IL-8, pg/ml	12.0 (7.1-21.6)	5.7 (4.3-11.0)	**<0.0001**

	(n=90)	(n=54)	
TNF-α, pg/ml	2.4 (1.8-4.3) (n=90)	1.7 (1.3-2.1) (n=54)	**<0.0001**
IP-10, pg/ml	152.6 (66.8-449.7) (n=90)	100.0 (61.1-169.6) (n=54)	**0.007**
MCP-1, pg/ml	365.7 (226.3-571.5) (n=90)	210.1 (163.2-306.9) (n=54)	**<0.0001**
IFN-γ, pg/ml	4.6 (2.4-8.8) (n=90)	2.3 (1.1-4.9) (n=54)	**0.001**
IFN-α, pg/ml	0.68 (0.29-0.87) (n=90)	0.40 (0.00-0.74) (n=54)	**0.02**
IFN-β, pg/ml	5.3 (4.3-6.7) (n=90)	5.3 (3.8-6.7) (n=54)	0.99
IFN-λ1, pg/ml	3.7 (2.3-5.0) (n=90)	2.4 (0.7-3.8) (n=54)	**0.003**
Abbreviations: Ang-1 and Ang-2=angiopoietin 1 and 2; hs-CRP=High-sensitivity C-Reactive protein serum; IL=interleukin; IFN=interferon; IP-10=interferon gamma-induced protein 10; MCP-1= Monocyte chemoattractant protein 1; SAA=amyloid A; sICAM-1 and sICAM-3=soluble intercellular adhesion molecule 1 and 3, sVCAM-1=soluble vascular cell adhesion molecule 1; TM=thrombomodulin;			

TNFRII=Tumour Necrosis Factor Receptor II; TNF-α=Tumour Necrosis Factor-α. All values are median (interquartile range) unless otherwise specified.

Table 4.1. Cytokines/chemokines and inflammatory molecules in patients with Juvenile dermatomyositis (JDM) and healthy controls*. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

4.5.1 TNFRII levels in children with JDM

As TNFRII has been previously suggested as a possible biomarker for monitoring disease activity in JDM, patients were then assessed for TNFRII levels based on disease activity. Patients with active disease had higher levels of TNFRII median of 6272 (IQR, 4246-11283) pg/ml when compared with patients with inactive disease with a median of 4803 (IQR, 3626-6840) pg/ml, but this difference did not reach statistical significance, p=0.076 (**Figure 4.1**).

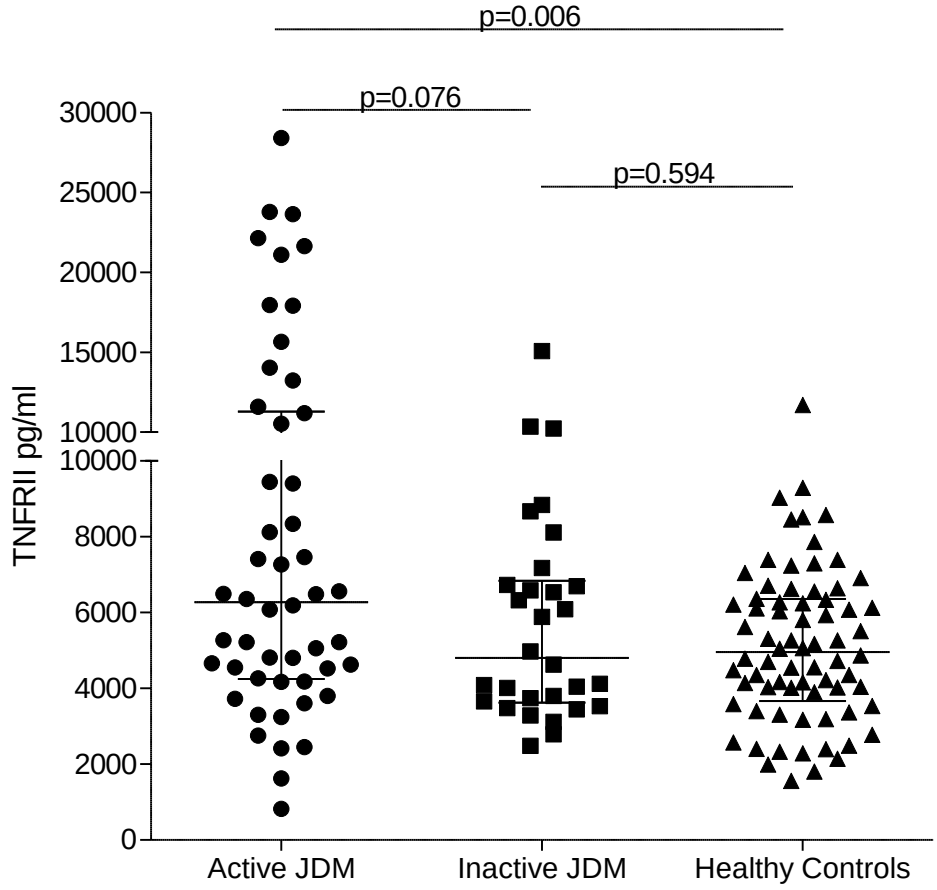

Figure 4.1. Tumour Necrosis Factor Receptor II (TNFRII) levels according to disease activity status in patients with Juvenile dermatomyositis (JDM) compared to healthy controls. *Children with JDM had higher plasma levels of TNFRII median 6258 (interquartile range, IQR, 4055-9433) pg/ml compared to healthy controls, median 5048 (IQR, 3748-6354) pg/ml, p=0.006. Children with active disease tended to have higher levels of TNFRII median 6272 (interquartile range, IQR, 4246-11283) pg/ml compared to children with inactive JDM median 4803 (IQR, 3626-6840) pg/ml, p=0.076. There was no difference in TNFRII levels between*

children with inactive JDM and healthy controls, however, p=0.594. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.

4.5.2 Galectin-9 levels in children with JDM

Galectin-9 levels were assessed in 90 patients with JDM and 82 healthy controls. Patients with JDM had higher levels of Galectin-9 median of 76.3 (IQR, 52.4-134.0) ng/ml when compared to healthy controls, with a median of 38.4 (IQR, 27.1-50.8) ng/ml, p<0.0001. When disease activity was taken into account, patients with active JDM had significantly higher values of Galectin-9 at a median of 108.6 (IQR, 61.15-219.0) ng/ml compared with patients with inactive disease with a median of 62.03 (IQR, 47.71-81.30) ng/ml, p=0.0008 (**Figure 4.2**). Patients with inactive disease on treatment had no significantly different serum levels of Galetin-9 when compared to JDM patient with inactive disease off treatment, p=0.58 (**Figure 4.3**). As Galectin-9 is mainly produced by endothelial cells, correlation of Galectin-9 with CEC was examined (**Figure 4.4**), while Galectin-9 levels was also examined in association to presence of nailfold capillaries changes (**Figure 4.5**). In the 25 patients studied prospectively, galectin-9 levels changed significantly when there was a change in disease activity status between the two studied time points (**Figure 4.6**).

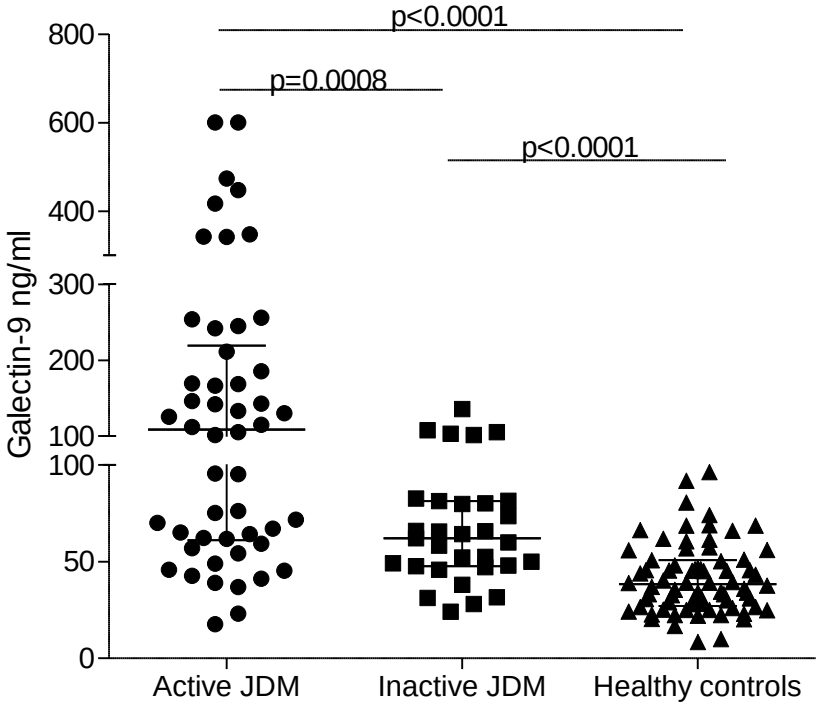

Figure 4.2. Levels of Galectin-9 according to disease activity status in Juvenile dermatomyositis (JDM) patients compared to healthy controls*. Children with active JDM had significantly higher levels of galectin-9 median 108.6 (interquartile range, IQR, 61.15-219.0) ng/ml compared to children with inactive disease median 62.03 (IQR, 47.71-81.30) ng/ml, p=0.0008, and healthy controls median 38.4 (IQR, 27.1-50.8) ng/ml, p<0.0001. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

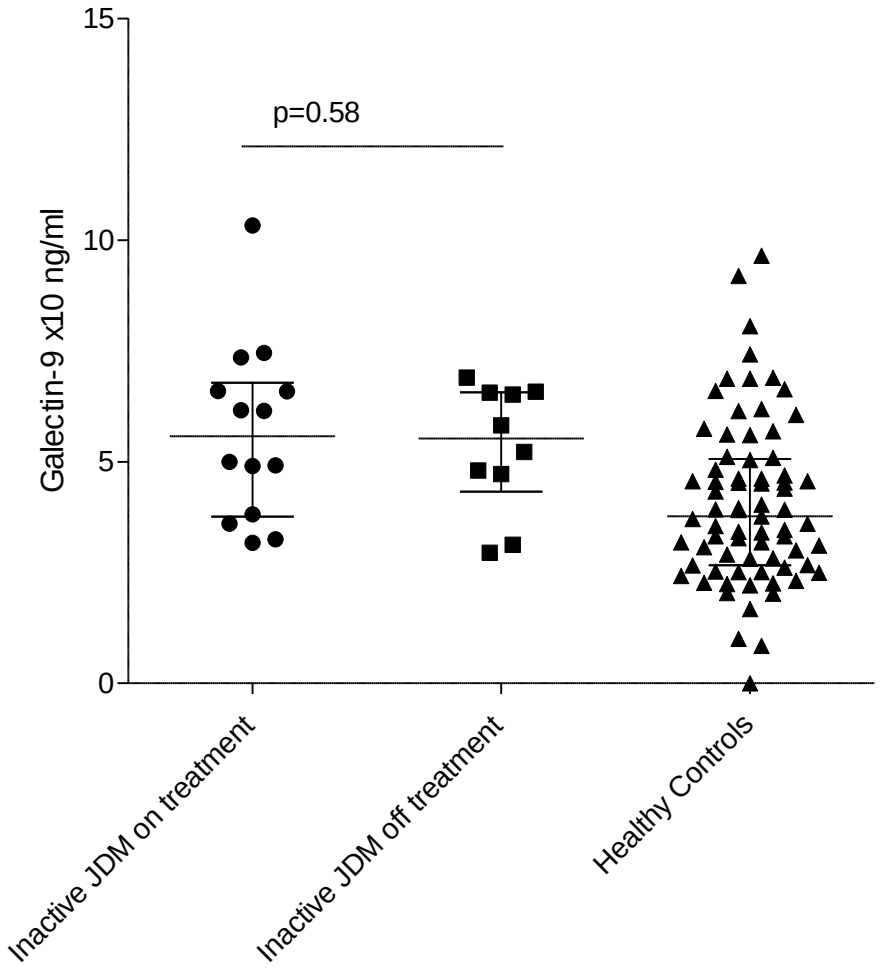

Figure 4.3. Galectin-9 levels in Juvenile dermatomyositis (JDM) patients with inactive disease. *Galectin-9 levels in inactive JDM patients still receiving treatment did not differ when compared to inactive JDM patients but who were off treatment, p=0.58. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

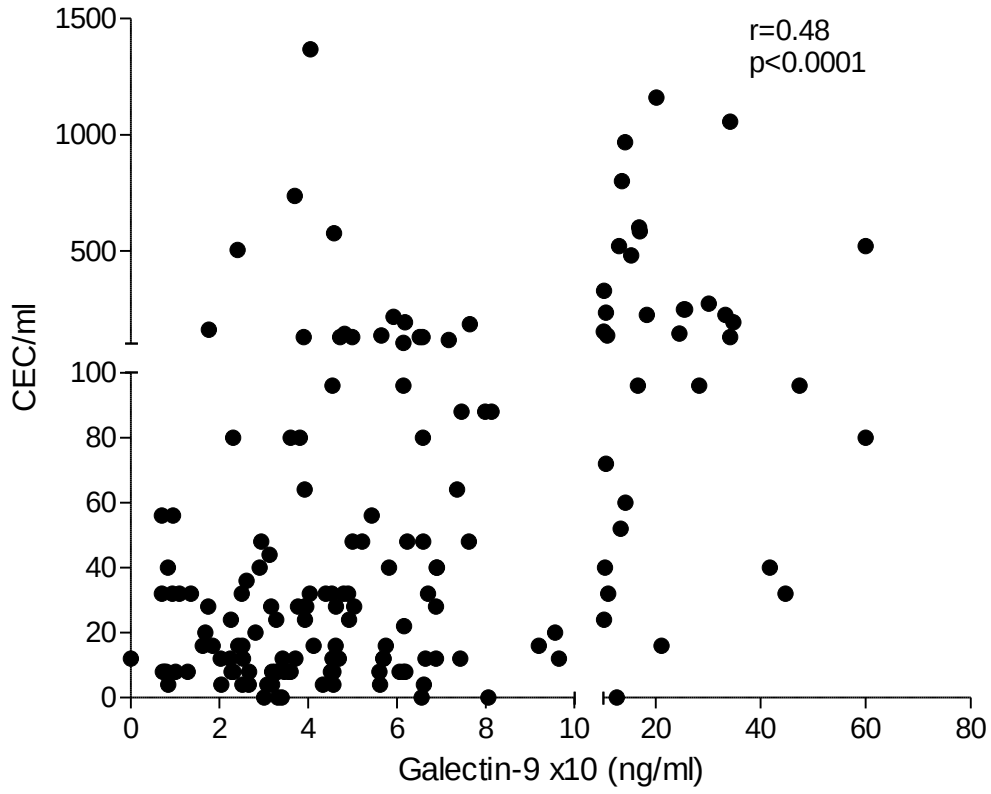

Figure 4.4. Galectin-9 levels strongly correlate to Circulating Endothelial Cells (CEC) levels in Juvenile dermatomyositis (JDM). *Spearman's correlation coefficient r=0.48, p<0.001.*

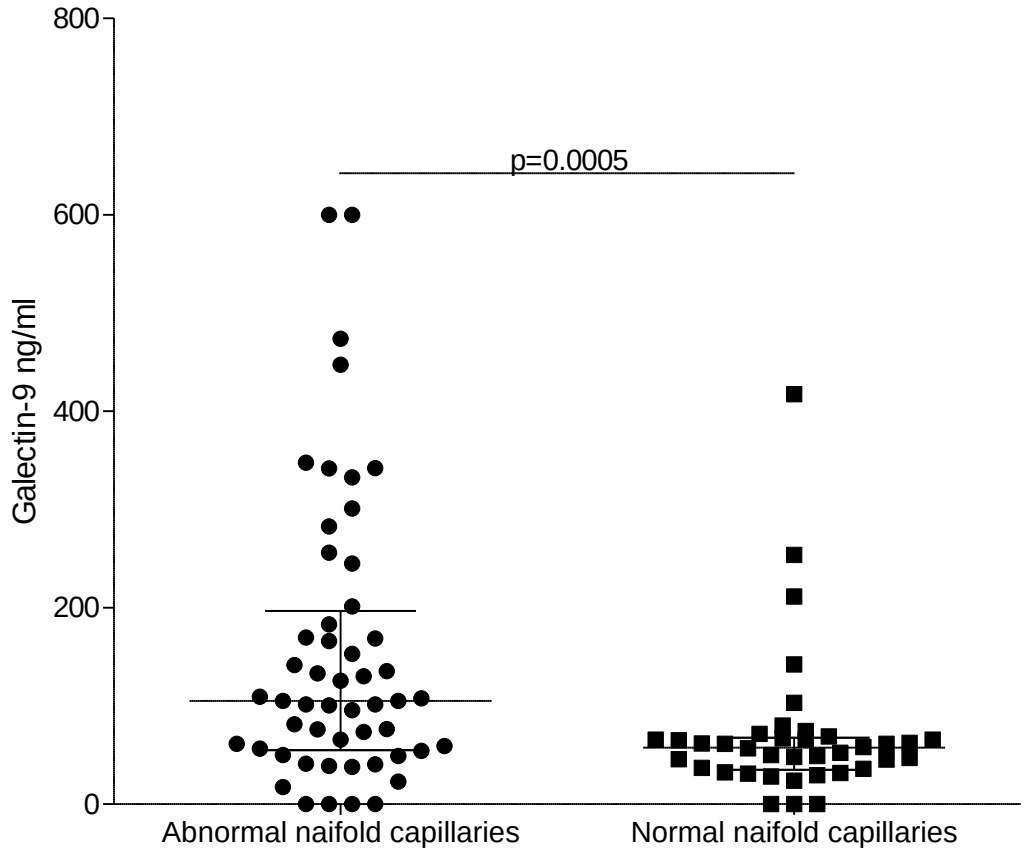

Figure 4.5. Galectin-9 levels in Juvenile dermatomyositis (JDM) patients according to the presence of abnormal nailfold capillaries. *JDM patients with abnormal nailfold capillaries had higher levels of Galectin-9 at a median of 105.1 (interquartile range, IQR, 54.8-196.7) ng/ml compared to patients with normal nailfold capillaries median 57.6 (IQR, 35.1-67.5) ng/ml, p=0.0005. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

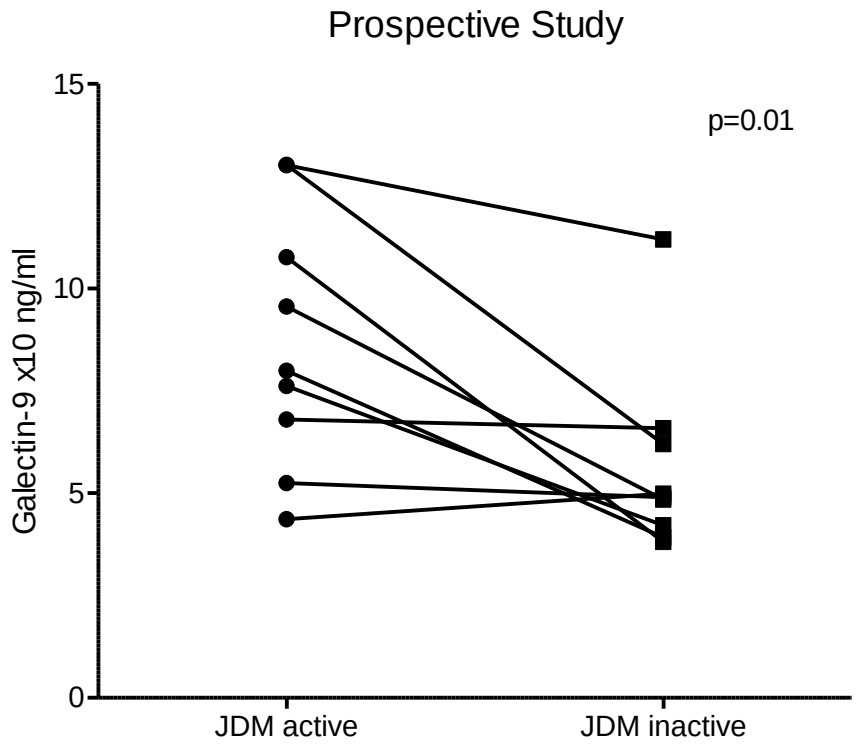

Figure 4.6. Prospective study of galectin-9 levels in children with Juvenile dermatomyositis (JDM). *Galectin-9 levels in JDM patients differ significantly when the patient's disease status changed from active to inactive, p=0.01, in the prospective part of this study. P values were calculated using Wilcoxon matched pairs signed rank test.*

Finally, as galectin-9 was found to be the best predictor of disease activity, the relationship between galectin-9 and commonly used tools for assessment of disease activity was investigated. Galectin-9 was found to strongly to correlate with PhyGLOVAS ($p<0.0001$), CMAS ($p<0.0001$) and MMT-8 ($p<0.0001$) (**Figure 4.7**).

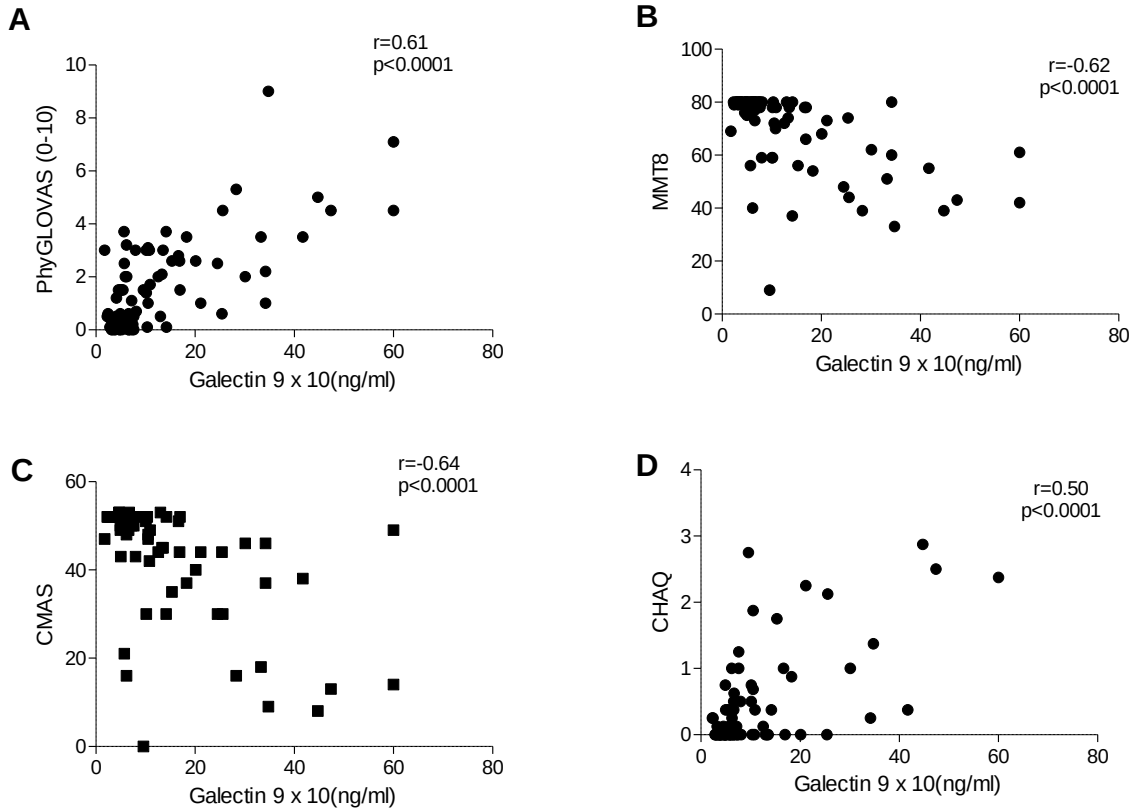

Figure 4.7 Correlation of serum Galectin-9 levels with scores of disease activity tools using non-parametric spearman's correlation test. *Associations were demonstrated with Physician Global Assessment in Visual Analogue Score (PhyGLOVAS, p<0.0001)* ***Figure 4.7A****, with Manual Muscle Testing -8 (MMT-8, p<0.0001)* ***Figure 4.7B****, with Childhood Myositis Assessment Score (CMAS, p<0.0001)* ***Figure 4.7C*** *and Childhood Health Assessment Questionnaire (CHAQ, p<0.0001)* ***Figure 4.7D****.*

4.5.3 Association of type I IFN signal with MSA

To determine whether type I IFN signal was associated with distinct MSA, serum levels of IFN-α, IFN-β, IFN-λ and galectin-9 were compared in the three main MSA subgroups of this JDM cohort; patients with positive anti-MDA5, anti-NXP2 and anti-TIF1g antibodies. Galectin-9 levels were also compared as in a recent study in SLE patients galectin-9 was suggested to reflect the IFN signature in the studied patients (van den Hoogen *et al.*, 2018a; van den Hoogen *et al.*, 2018b). The type I IFN signature tended to be higher in patients with MDA5 positive antibodies compared to patients with NXP2 and TIF1g antibodies as demonstrated by higher levels of IFN-α, IFN-β, IFN-λ and galectin-9, respectively (**Figure 4.8**), but these results did not reach statistical significance probably due to the low numbers of included patients.

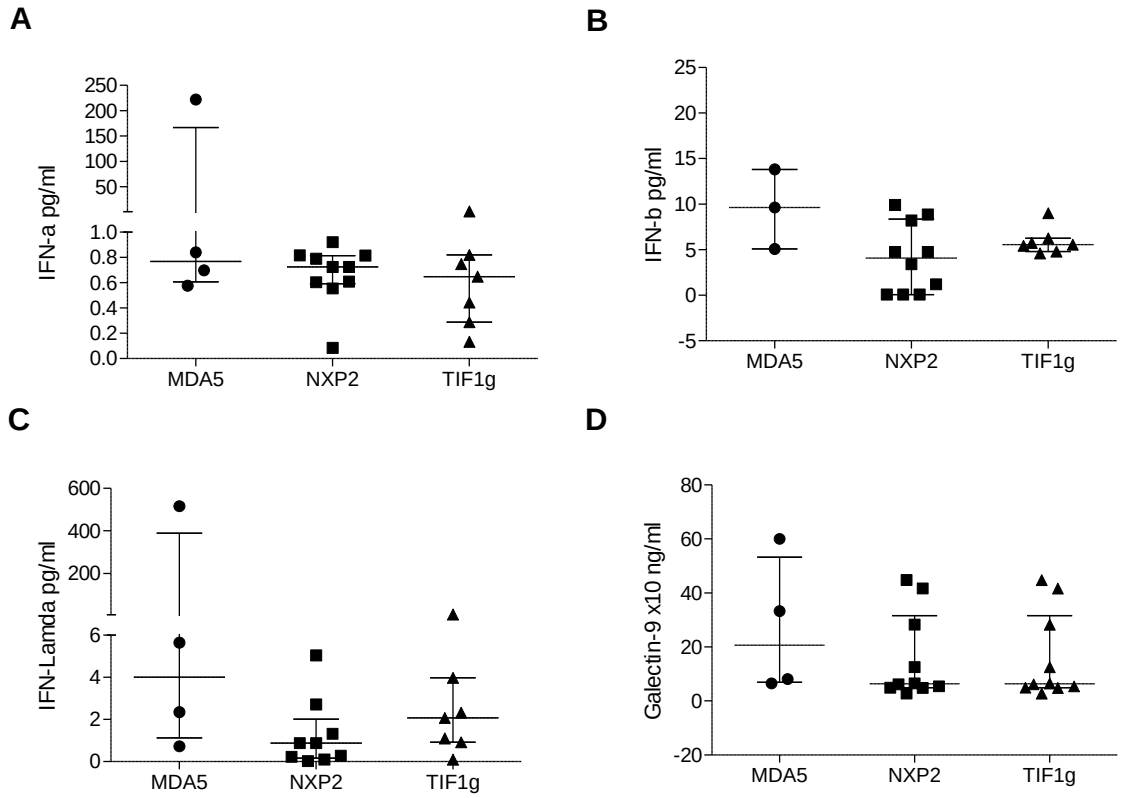

Figure 4.8. Type I interferon (IFN) signature in Juvenile dermatomyositis (JDM) patients based on their myositis specific antibody (MSA) status. *Children with positive melanoma differentiation-associated protein 5 (MDA5) antibodies tended to have higher levels of IFN-α (**Figure 4.8A**), IFN-β (**Figure 4.8B**)*, IFN-λ (**Figure 4.8C**) and galectin-9 (**Figure 4.8D**) compared to children with positive nuclear matrix protein 2 (NXP2) and transcription intermediary factor 1-gamma (TIF1g) antibodies. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range. * Serum levels of IFN-β could not be measured in the serum of a child with positive MDA5 antibodies for technical reasons.*

4.5.4 Galectin-9 and TNFRII levels for monitoring disease activity

To further assess the test characteristics of these biomarkers for prediction of disease activity, receiver operating characteristic (ROC) curves were created to calculate predictive values, sensitivity, and specificity, as listed in **Table 4.2**. Patients with active disease were compared with patients with clinically inactive disease. For TNFRII and Galectin-9 the area under the curve (AUC) was 0.619 and 0.724, respectively. For the calculation of predictive values, several cut-off values were used for both studied biomarkers to achieve a specificity of at least 80%, as follows: for Galectin-9 107.7 ng/ml and for TNFRII 7175.9 pg/ml. This returned a specificity of 96.8% and 80% and a sensitivity of 50% and 42%, respectively. Galectin-9 was also shown to strongly associate with active disease with a positive likelihood of 15.50 compared to a positive likelihood of 2.10 for the TNFRII. Thus, Galectin-9 was shown to be a better biomarker for disease activity compared to TNFRII.

Test characteristic	**Galectin-9**	**TNFRII**
AUC (95% CI)	0.724 (0.613-0.817)	0.619 (0.504-0.726)
Cut-off values	>107.7 ng/ml	>7175.9 pg/ml
+LR	15.50	2.10
-LR	0.52	0.72
Sensitivity (95% CI)	50 (35.5-64.5)	42.0 (28.2-56.8)
Specificity (95% CI)	96.8 (83.2-99.5)	80 (61.4-92.2)

AUC = Area under the curve; 95% CI = 95% confidence interval +LR = positive likelihood ratio; -LR = negative likelihood ratio; TNFRII = Tumour Necrosis Factor Receptor II

Table 4.2. Positive and negative likelihood ratio, sensitivity, specificity and area under the curve for Galectin-9 and Tumour Necrosis Factor Receptor II (TNFRII) in patients with active and clinically inactive Juvenile dermatomyositis (JDM).

4.6 Discussion

There is a lack of biomarkers validated and used in clinical practise for JDM. That inevitably complicates clinical management, hampers clinical trial design, and ultimately the development of new treatments. Moreover, there is currently no laboratory test that can reliably predict, identify and monitor an episode of disease flare related to the vasculopathy of JDM and response to treatment; and no tool that can guide timing of treatment discontinuation. Therefore, there has been an increasing interest for several laboratory markers that could serve as a biomarker of disease activity, to stratify treatment and monitor the response, to detect flares, to guide when to stop treatment, and gauge overall prognosis. Moreover, such an (aspirational) biomarker should be relevant to the pathophysiology of the disease, be easy to use in clinical practice, and (ideally) should correlate to traditionally used indices for monitoring disease activity status (Illei *et al.*, 2004).

As demonstrated in this study, a number of cytokines/chemokines and adhesion molecules are elevated in patients with JDM. Some of the studied parameters are mainly expressed by endothelial cells and are highly indicative of endothelial activation. The association of endothelium-derived soluble adhesion molecules with JDM is in keeping with the results in chapter 3 suggesting that endothelial injury and dysfunction plays a significant role in the pathophysiology of JDM. ICAM-1, V-CAM 1 and E-selectin were significantly higher in JDM patients compared to healthy controls. The upregulation of adhesion molecules has been previously reported in cutaneous lesions in DM (Kumamoto *et al.*, 1997). VCAM-1 and E-selectin are mainly secreted from activated endothelial cells, highlighting the association of these molecules with JDM vasculopathy. The soluble forms of these molecules maintain most of the functions and the structure of the cell-bound adhesion molecules. It would be of great interest to further investigate their potential therapeutic role. Indeed, an anti-adhesion molecule monoclonal antibody (efalizumab, a humanised anti-CD11a monoclonal antibody) has been proposed as a new therapeutic agent in patients with psoriasis (Dedrick *et al.*, 2002).

Several studies in DM have found elevated cytokine levels (Gono *et al.*, 2014), but in children their role has not been extensively investigated. Of interest, in two studies looking into the pattern of cytokine production by PBMC in DM patients, Th2 cytokines including IL-10 and IL-6 were only occasionally detected in very few cells leading the authors to the conclusion that these cytokines are probably secreted early in the disease course (Hagiwara *et al.*, 1996; Lundberg *et al.*, 1997). My study demonstrated increased levels of IL-6 and IL-10 regardless of disease duration, as the study population had a median disease duration of 1.63 (0.28-4.66)

years. IL-6 and IL-10 have also been suggested as possible biomarkers for monitoring disease activity in patients with ILD related to DM compared to those without ILD (Kawasumi *et al.*, 2014; Nara *et al.*, 2014). My study demonstrated increased level of both IL-6, IL-8 and IL-10 in JDM patients but it was not possible to study their correlation with the development of ILD due to the small numbers (only 6 patients had ILD at the time of recruitment, and none of the recruited patients had rapidly progressive ILD). IL-6 was found to be a mediator of inflammation in a mouse model of myositis (Scuderi *et al.*, 2006). A report published in 2011 of 2 adults with refractory disease suggested tocilizumab as a possible therapeutic regime (Narazaki *et al.*, 2011). Currently in the United States, an ongoing clinical trial is looking at the efficacy of IL-6 blockade with tocilizumab in refractory DM cases (ClinicalTrials.gov Identifier: NCT0204354).

TNF-α was significantly elevated in JDM patients compared to healthy controls. This finding is in keeping with previous studies demonstrating that TNF-α is implicated in disease pathogenesis in inflammatory myopathies as increased levels of TNF-α have been found in muscle biopsies of patients with DM (Kuru *et al.*, 2003) along with increased serum levels (Werth *et al.*, 2002). Moreover, levels of TNF-α have been shown to correlate with type I IFN signature in myositis patients (Higgs *et al.*, 2012). Anti-TNF-α biologic agents are a potential therapeutic alternative to standard treatment for JDM, as TNF-α has been shown to cause dysfunction and increased catabolism in muscle fibres; and blockade of myogenesis (Li and Reid, 2001; Efthimiou, 2006). That said, evidence on anti-TNF treatment in DM so far has been contradictory. Infliximab, a chimeric monoclonal antibody against TNF-α was (in a

retrospective series) considered effective in children with refractory JDM (Riley *et al.*, 2008). Results from two open-label study in adults, however, have been inconclusive due to high flare rates (Dastmalchi *et al.*, 2008; Hengstman *et al.*, 2008). On the other hand, treatment with adalimumab and etanercept for other autoimmune diseases has actually induced inflammatory myopathy is some cases (Ramos-Casals *et al.*, 2007; Nagashima and Minota, 2011). Currently, a clinical trial of infliximab for the treatment of refractory DM is underway (ClinicalTrials.gov NCT00033891) (Schiffenbauer *et al.*, 2018).

In this thesis, the IFNs and IFN related protein profile was also studied. IFN-α, IFN-λ1 and IFN-γ were significantly increased in JDM patients compared to healthy controls; there was no difference in the levels of IFN-β levels. Similarly, IFN related chemokines, MCP-1 and IP10, were both increased in JDM patients compared to healthy controls. Interestingly, children with MDA5 positive MSA tended to have a stronger type I IFN signature as demonstrated by elevated serum levels of IFN-α, IFN-β, IFN-λ and galectin-9. These findings are in keeping with a previous study in adults DM patients, in that there was an association between the DM subgroup with autoantibodies against RNA-binding proteins and type I IFN signature, perhaps suggesting a pathogenic role of these MSA for the induction of type I IFN. MDA5 is a double stranded (ds) RNA helicase enzyme that in humans is encoded by the *IFIH1* gene (Silva *et al.*, 2016). It is now well described that the type I IFN pathway plays a crucial role in JDM pathogenesis, and is seen upregulated in both muscle (Tezak *et al.*, 2002; Chen *et al.*, 2008), skin (Wenzel *et al.*, 2006; Lopez de Padilla *et al.*, 2007; Shrestha *et al.*, 2010), and peripheral blood cells (Baechler *et al.*, 2007). Studies of type I IFN levels in general have been hampered

by technical difficulties of currently available immunoassays which have relatively low sensitivity. This has spurred the development of proxy assays such as IFN related gene expression that is extensively used in several studies. Although, results are highly suggestive of upregulation of type I IFN pathway in JDM, the use of different combinations of IFN related genes in the different studies have made comparison of the results challenging. Recently, a highly sensitive single molecule array (SIMOA) for the measurement of serum levels of total IFN-α has been described which enabled the direct quantification of IFNα at attomolar (femtograms per millilitre) concentrations (Rodero *et al.*, 2017). In contrast, the assay used in my thesis (Mesoscale Discovery) was able to detect IFN-α2a levels in pg/ml, thus accurate quantification of IFN-α levels in many healthy controls was impossible due to lower levels in health, below the MSD assay detection threshold.

Interestingly, apart from the type I IFNs, IFN-γ was also significantly increased in JDM patients compared to healthy control suggesting a possible role of the type II IFN pathway in disease pathogenesis. IFN-γ is expressed by multiple immune cells and prepares macrophages and dendritic cells to respond to Toll Like Receptors (TLR) signals (Schroder *et al.*, 2004). It is connected with the expression of several chemokines including IP10 which has also been elevated in my patient cohort in keeping with previous studies (Bellutti Enders *et al.*, 2014). A recent study in SLE patients demonstrated elevated levels of IFN-γ and the chemokine IP-10 in preclinical SLE patients prior to IFN-α activity and to positivity for most autoantibodies (Munroe *et al.*, 2016). It would be of great interest to investigate serum levels of IFN-γ in JDM patients very early in the disease course in correlation with type I IFN levels and antibodies.

Interestingly, Ishikawa *et al*. demonstrated increased serum levels of IFN-γ in DM patients with rapidly progressive ILD suggesting a possible pathogenetic role of IFN-γ through macrophage activation and acceleration of inflammation (Ishikawa *et al.*, 2018). Thus, there may be a role of anti-IFN-γ agents in the treatment of JDM, especially when complicated with ILD.

IFN-λ1 belongs to a newly characterised IFN family, the type III IFNs which also includes IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). IFN-λs act through binding with IFN-λ Receptor 1 and 2 which subsequently trigger the Janus Kinase (JAK)/Signal transducer and activator of transcription (STAT) cascade in a similar way with the receptor for type I IFN (Iversen and Paludan, 2010) leading to the conclusion that type III IFNs may share similar biological activities with type I IFNs (Donnelly and Kotenko, 2010). Apart of the similarities between IFN-λ and IFN-α, there are a lot of differences between the two IFN families. IFN-α is mainly produced by peripheral dendritic cells while IFN-λ is produced by a wide variety of cells (Iversen and Paludan, 2010). IFN-λ expression mainly relates to nuclear factor-kappa B (NF-κB) activation independent of the IFN regulatory factor suggesting a possible induction of numerous stimuli (Iversen and Paludan, 2010). Finally, IFN-α targets a large number of cells, in contrast with IFN-λ which is restricted mainly to epithelial cells (Sommereyns *et al.*, 2008) In SLE patients, IFN-λ3 has been associated with the extend of disease activity (Amezcua-Guerra *et al.*, 2017) but this is the first time studied in JDM patients. In my study, IL-λ1 levels were found to be significantly elevated in JDM patients compared to healthy controls suggesting a possible new pathway contributing to disease pathogenesis. This needs to be further investigated in a larger study.

TNFRII and, especially, Galectin-9 were found to link to disease activity levels. Patients with active disease had almost two times higher levels of galectin-9 compared to the ones with clinically inactive disease. Moreover, galectin-9 was found to be significantly elevated in patients with abnormal nailfold capillaries. It has been previously demonstrated that persistent nailfold abnormalities are indicative of persistent active vasculopathy and could potentially identify patients at high risk of developing long-term complications and severe organ damage (Schmeling *et al.*, 2011). Results are in keeping with a previously published Dutch study where 25 patients with JDM were included (Bellutti Enders *et al.*, 2014) while for the first time galectin-9 has been associated with other markers of endothelial injury. In 2018, a study has been published suggesting that Galectin-9 reflects the IFN signature in SLE patients that raised several comments and made the authors to suggest that the use of galectin-9 as a marker of IFN activity needs further confirmation before clinical implementation (van den Hoogen *et al.*, 2018a). Since Galectin-9 and CEC are both markers of disease activity, one could suggest that when combined with nailfold capillaroscopy findings they provide some robust biomarkers to indicate ongoing vasculopathy in some JDM patients even when no clinical signs of muscle disease or other skin rashes are present. On the other hand, levels of these biomarkers could guide clinicians' decisions about stopping treatment in patients with clinically inactive disease. Moreover, the combination of these biomarkers in newly diagnosed patients may serve as a predictor of disease severity and disease activity duration. A survival analysis along with a large multicentre prospective study may give further information and shed light into this aspect.

To summarise, this cross-sectional study has shown elevated levels of soluble adhesion molecules and proteins related to endothelial activation and subsequent increased endothelial damage in patients with JDM. Galectin-9 and TNFRII, in particular, could serve as biomarkers of disease activity and could further guide therapeutic decisions in patients with clinically inactive disease.

5 Circulating microparticles in children with Juvenile Dermatomyositis

5.1 Summary

Background: MP are membrane vesicles released during cell activation or apoptosis that can allow tracking of endothelial injury and cellular activation. MP also have important procoagulant properties. MP have never been studied before in JDM.

Objectives: To investigate the MP profiles associated with endothelial activation and cellular activation in children with JDM, and to examine whether this approach could serve as a biomarker for monitoring the vasculopathy of JDM.

Methods: 90 patients recruited to the UK JDM Cohort & Biomarker Study were included; median age 10.21 (IQR, 6.68-13.40) years with median disease duration of 1.63 (IQR, 0.28-4.66) years. Inactive disease was defined as per modified PRINTO criteria: no skin rashes, CK≤150 U/l, CMAS≥ 48/52, MMT8≥78/80, PhyGLOVAS ≤0.2/10 on a visual analogue scale. MP were identified with multicolour flow cytometry.

Results: Total circulating MP counts differed significantly between those with active JDM, median 301 (IQR, 186-584) $x10^3$/ml and those with inactive disease, median 81 (IQR, 34-191) $x10^3$/ml, $p<0.0001$; and controls 44 (15-249) $x10^3$/ml, $p<0.0001$. These circulating MP were predominantly of platelet and endothelial origin. Total AnV+ MP counts were strongly correlated with circulating endothelial cells ($r=0.42$, $p<0.0001$) and strongly correlated with the PhyGLOVAS ($r=0.43$, $p=0.0002$)

Conclusion: Circulating MP profiles may reflect distinct disease activity status in patients with JDM. These novel biomarkers of vascular pathology, platelet activation and thrombotic propensity can track disease trajectories in JDM and predict high risk groups for poorer disease outcomes.

5.2 Introduction

The development of autoimmunity is considered to be the result of a combination of genetic factors, environmental triggers, and defects in mechanisms that regulate the immune system (Wedderburn and Rider, 2009). Both adaptive and innate immunity have an important role in the initiation and continuation of immune responses. The formation of autoantibodies, defects in autoregulatory functions, and the activation of T-cells (Czompoly *et al.*, 2006) along with the release of soluble factors, autoantigen presentation and effector T-cell activation (Segelmark, 2011) are implicated in the pathogenesis of autoimmune diseases. In addition, defects in cell death pathways and clearance of apoptotic debris have also been implicated in autoimmunity (Munoz *et al.*, 2010). Defects in apoptotic mechanisms result in overproduction of autoantibodies through survival of autoreactive B-cells (Rahman, 2011), while defects in removal of apoptotic debris may turn them into a new source of autoantibodies and immune complexes (Burbano *et al.*, 2015).

5.2.1 Circulating MP

Circulating MP are small membrane derived vesicles (100 nm to 1µm in size) which are

released upon activation of the cells (including endothelial cells) and/or apoptosis (Brogan *et al.*, 2004). MP contain a wide variety of molecules providing them with the ability to regulate endothelial cell activation and proliferation (Klinkner *et al.*, 2006), coagulation and thrombosis (Aharon *et al.*, 2008) and inflammation. They originate from different cells, and thus contain several components derived from the parent cell of origin. They are rich in aminophospholipids, particularly phosphatidylserine (PS) (Morel *et al.*, 2004; Dignat-George and Boulanger, 2011). Their composition also differs depending on the trigger that generated their formulation and release (Dignat-George and Boulanger, 2011). Notably MP have important prothrombotic properties due to the provision of a large PS surface area for the assembly and activation of coagulation enzyme complexes, the expression of tissue factor (TF) and the ability to activate platelets in the vicinity of thrombus formation (Falati *et al.*, 2003). Approximately 80% of circulating MP arise from platelets (Flaumenhaft *et al.*, 2009) and 5-15% from endothelial cells known as EMP (Pericleous *et al.*, 2009). MP belong to a highly heterogenous group of extracellular vesicles, which can be broadly divided into two main categories: exosomes and microvesicles (**Table 5.1**) (van Niel *et al.*, 2018). Microvesicles are formed by budding of the plasma membrane (Cocucci and Meldolesi, 2015). On the other hand, exosomes are developed from the in-budding of endosomes, which then form multi-vesicular bodies that contain intra-luminal vesicles (Cocucci and Meldolesi, 2015). The multi-vesicular bodies then fuse with the cell membrane and release the intra-luminal vesicles into the circulation as exosomes (Sarko and McKinney, 2017). MP differ from exosomes and apoptotic bodies (two of the most commonly described vesicular structures) in size, composition and circulating numbers (Thery *et al.*, 2009) (**Table 5.2**).

	Exosomes	Microvesicles
Origin	Endosome	Plasma membrane
Size	50-150 nm	50-500 nm (up to 1 μm)
Other names (according to their origin, size and morphology)	Prostasomes Tolerosomes Dexosomes Nanovesicles Exosome-like vesicles and others	Microparticles Blebbing vesicles Shedding vesicles Oncosomes ARRMs Migrasomes Neurospheres Apoptotic bodies

Table 5.1. Types of extracellular vesicles. *Extracellular vesicles are divided in two main categories: exosomes and microvesicles based on their size, origin, morphology and content.*

Feature	Exosomes	Microparticles (MP)	Apoptotic cells
Size	40–100 nm	100–1000 nm	>4000 nm
Membrane of origin	Multivesicular endosomes	Plasma membrane	Plasma membrane
Generation	Spontaneous release and cellular activation	Spontaneous release, cellular activation, and apoptosis	Apoptosis
Annexin V binding	Low or negative	High, low, or negative	High
Functions	Carrying lytic enzymes and activation of	Coagulation, M2 macrophage activation, and transfer of	Antigen presentation through MHC II, M2 macrophage and

	phagocytes and B-cells	functional cell components	monocyte activation and tissue remodelling
Markers	Rab GTPases, annexins, flotillin, Alix, TSG101, and CD63	Integrins, selectins, proteins from the parental cells, and phosphatidylserine	Histones, phosphatidylserine
Organelles		Platelet MP might contain mitochondrial structures	Different
Nucleic acids	No	mRNA, DNA, miRNA, and interfering RNA	DNA, mRNA, and miRNA

Table 5.2. Characteristics and properties of the main secreted vesicles. *Adapted from Bulbano et al* (Burbano *et al.*, 2015).

The following sections give an overview of MP generation and composition, identification and enumeration methods and their role in autoimmune and other diseases.

5.2.2 MP generation and composition

Environmental and physiological triggers can induce cell differentiation, division, and structural changes in membrane cytoskeleton promoting MP generation (Hugel *et al.*, 2005; Gomes *et al.*, 2006). Two pathways that have been involved in MP formulation include changes in the membrane; and in the cytoskeleton of the cells (Cauwenberghs *et al.*, 2006; Gao *et al.*, 2012). Both these events are dependent on the levels of intracellular calcium. As

calcium is involved in numerous complex pathways, the exact mechanism of MP formulation is not fully understood.

The cell membrane is composed by a lipid bilayer, with the inner leaflet differing from the external one in aminophospholipid composition. The inner side is rich in phosphatidyl-ethanolamine (PE) and PS; while the outer layer is rich in sphingomyelin and phosphatidylcholine (PC) (Hugel *et al.*, 2005). The composition of the bi-lipid membrane is controlled by adenosine triphosphate (ATP)-binding transport enzymes, such as floppases (that transport lipids to the outer layer); and flippases (that transport lipids to the inner layer) (Zwaal *et al.*, 2005). When calcium intracellular concentration is increased, flippase activity is inhibited, and floppase activity is upregulated resulting in PS transportation to the outer membrane leaflet. Membrane aminophospholipid composition is further controlled by bidirectional lipid transporters, the scramblases which are also activated by increased intracellular calcium levels (Morel *et al.*, 2011). When the balance of the lipid composition is disturbed along with reorganisation of the cytoskeleton and the action of calcium dependent proteolytic enzymes (calpains), the cell membrane shrinks, causing the formation of MP. Surface exposure of PS that happens transiently during cell activation (Proulle *et al.*, 2005) and permanently during cell apoptosis and necrosis (Cauwenberghs *et al.*, 2006; Gao *et al.*, 2012) is associated with MP release (Zwaal *et al.*, 2005). Another important regulator of MP generation is lipid rafts. Lipid rafts are sterol and sphingolipid-enriched membrane domains that act as organising centres that promote aggregation of signalling molecules that influence

membrane fluidness and transportation of membrane proteins (Korade and Kenworthy, 2008; Pike, 2009).

Several studies have focused specifically on investigating the mechanisms of EMP formulation. Using gene expression profiling, Sapet *et al.* identified an original mechanism that induce MP generation from endothelial cells depending on thrombin dependent activation of endothelial cells and involving activation of the Rho-kinase ROCK-II (Sapet *et al.*, 2006). Interestingly, ROCK-II was found to be activated by caspase 2 in the absence of cell death suggesting a mechanism of MP formulation during cell activation and not cell apoptosis (Sapet *et al.*, 2006). Other studied stimuli that contribute to MP release from endothelial cells include TNF-α (Liu *et al.*, 2013; Yamamoto *et al.*, 2015), IL-1, IFN-γ, bacterial lipopolysaccharide (Yamamoto *et al.*, 2015), CRP (Wang *et al.*, 2007) and plasminogen activator inhibitor-1 (PAI-1) (Brodsky *et al.*, 2002). Apart from these pro-inflammatory stimuli, hypoxia also seems to promote the release of EMP, but results have been controversial with some studies suggesting that hypoxia induced EMP release is mainly associated with endothelial activation (Lichtenauer *et al.*, 2015; Pichler Hefti *et al.*, 2016).

MP composition demonstrate high heterogeneity depending on the parental cell composition, the status of the cellular source, and the stimuli that triggered their release (Rubin *et al.*, 2008; Howes, 2010; Capriotti *et al.*, 2013; Liu *et al.*, 2013). Deoxyribonucleic acid (DNA), messenger ribonucleic acid (mRNA) and micro-RNA has been identified in MP produced during cell apoptosis (Thery *et al.*, 2009); conversely, MP generated during cell

activation or at early stages of apoptosis do not possess DNA (Burbano *et al.*, 2015). Interestingly, platelet derived MP (PMP) may carry functional mitochondria, which can then be transferred to neutrophils contributing to inflammation promotion, or to act as antigens for autoantibody generation (Boudreau *et al.*, 2014). Proteomic studies of EMP have indicated that the protein component of EMP has dual effect: one is a regulatory function of target cells and the second is a protective function as harmful proteins are released from the parental cell (Jimenez *et al.*, 2003; Peterson *et al.*, 2008; Liu *et al.*, 2013).

5.2.3 Identification and enumeration of MP

Several techniques are used for the identification and enumeration of MP, including electron microscopy, flow cytometry, ELISA, nanoparticle tracking analysis (NTA), and Western blotting. Electron microscopy was the first technique used. In a typical protocol cells are cultured on glass slides, fixated, dehydrated by mixing with different concentrations of ethanol and covered with gold before examination (Combes *et al.*, 1999). Electron microscopy provides information not only for the presence of MP but also for the size and morphology, but cannot quantify MP. Moreover, it is labour-intensive and time consuming, and thus other methods are now more commonly used. ELISA is considered by some investigators an easier and more reproducible method, but it is limited by the fact that it detects any faction expressing PS, without being able to discriminate between exosomes, MP or apoptotic bodies (Yun *et al.*, 2016). Western blotting cannot determine the number of MP as it only allows detection of particle specific proteins (Witwer *et al.*, 2013).

Flow cytometry is the most commonly used technique for MP detection. It can differentiate particles based on their size (roughly equating to forward scatter characteristics), but also based on their cell origin as antibody-conjugated fluorophores are used that target distinct cellular antigens (Dignat-George and Boulanger, 2011). It is mainly used for MP detection from blood and other fluid samples. Before the analysis, latex beads are used for the set-up of size-based gating strategies. Multi-colour flow cytometry is used for the synchronous identification of MP of different cellular origin including endothelial cells, platelets, monocytes, B- and T-cells (Mobarrez *et al.*, 2010). Currently, AnV is the most commonly used marker for the detection of MP. AnV specifically binds to negatively-charged phospholipids (PS) that is highly expressed in the external surface of MP, although this too may not be ideal: recently, there are several reports of AnV negative MP (Connor *et al.*, 2010; Macey *et al.*, 2011). One report suggested that only 24% of EMP bind to AnV (Sekula *et al.*, 2011). Whether, that finding truly represents MP that do not express PS needs to be further elucidated. Another limitation of some flow cytometry techniques is the inability to properly distinguish particles of smaller size (< 0.5μm), due to the detection ability of each flow cytometer. Finally, flow cytometry detection ability is restricted by protein aggregates (Witwer *et al.*, 2013). Thus far, it has been demonstrated that the use of different methods of analysis makes comparison of different studies challenging (Maas *et al.*, 2017). In an effort to overcome these obstacles, the International Society of Extracellular Vesicles has published a guideline with necessary requirements for sample collection, MP isolation and analysis to enable comparisons between different studies (Witwer *et al.*, 2013; Lotvall *et al.*, 2014).

A novel method complementing flow cytometry is NTA, which enables characterization of smaller particles (up to 30 nm). NTA uses light scattering, which is a technique that does not require outside calibration standards. Based on the random motion of MP in the blood (Brownian motion), and using the Stokes-Einstein equation (a particle size is inversely correlated to its diffusion), the number and size of particles can then be calculated (Dragovic *et al.*, 2011; Gardiner *et al.*, 2013; Witwer *et al.*, 2013). Nanoscale fluorescence activated cell sorting (nanoFACS) is a rapidly emerging new method for the analysis and sorting of different size particles (Morales-Kastresana *et al.*, 2017). Imaging flow cytometry constitutes a combination of conventional flow cytometry with high resolution imaging enabling accurate measurement of both small and large particles by capturing each single particle while passing through the flow cell (Headland *et al.*, 2014; Erdbrugger and Lannigan, 2016)

5.2.4 MP and autoimmune diseases

As previously described, MP are formed either as a result of cell activation or damage and play a significant role in inflammation, thrombosis, angiogenesis and in the control of vascular tone (Morel *et al.*, 2007). MP have strong prothrombotic properties, mainly secondary to the presence of PS, which is necessary for the assembly of clotting enzyme complexes, and TF, which initiates the coagulation cascade (Roos *et al.*, 2010). In addition, MP represent a source of immunologically active molecules with an effect on various cells that can initiate a number of different processes such as inflammation, apoptosis and antigen presentation. Depending on the cell of origin, the target cell and the microenvironment that events are taking place, MP can initiate or upregulate inflammatory processes through several mechanisms including

cytokine upregulation, initiation of the complement cascade, transfer of cell surface receptors and increased leukocyte migration (Distler *et al.*, 2006). Finally, MP are important circulating signal components in the cell to cell interaction transferring their contents either via internalization or via interaction with surface receptors of the targeted cells (Distler *et al.*, 2006). In conclusion, MP display diverse pro-inflammatory and pro-coagulant activities suggesting a potential role in the pathogenesis of autoimmune diseases but also highlighting them as potential biomarkers of disease activity and response to treatment in this category of diseases.

MP have been mainly studied in SLE and RA in adults. Patients with RA have been found to have increased levels of MP even early at the disease onset when compared to healthy controls (Knijff-Dutmer *et al.*, 2002; van Eijk *et al.*, 2010). Increased levels of MP have been identified both in the blood but also in the synovial fluid (Biro *et al.*, 2007). They are produced by a number of different cells including B- and T-cells, platelets and monocytes (Berckmans *et al.*, 2001; Boilard *et al.*, 2010). They seem to play an important role in the pathogenesis of inflammatory arthropathy through different mechanisms. For example, PMP produced by collagen stimulation enhance the production of IL-8 and IL-6 (Boilard *et al.*, 2010) by fibroblast-like synoviocytes. In SLE patients, proteomic studies have demonstrated that MP are rich in immunoglobulins and complement proteins (Ostergaard *et al.*, 2013) suggesting a potential role in disease pathogenesis. Regarding the numbers of AnV (+) MP, studies have thus far given contradictory results. In 2009, Sellam *et al.* found increased number of AnV (+) MP in SLE patients compared to healthy controls, which was mainly attributed to enhanced

PMP production (Sellam *et al.*, 2009). In 2011, Nielsen et al. found decreased levels of AnV (+) MP when SLE patients were compared to healthy controls (Nielsen *et al.*, 2011). Interestingly, in the same study SLE patients had higher numbers of AnV (-) MP which were also correlated with disease activity and development of specific clinical features (Nielsen *et al.*, 2011). As SLE has been associated with features of vascular disease, EMP have been associated with endothelial dysfunction and vascular damage in patients with active disease (Parker *et al.*, 2014). In SSc, another autoimmune disease characterised by microvascular injury (Sgonc *et al.*, 1996), studies on EMP and PMP have given conflicting results (Guiducci *et al.*, 2008; Iversen *et al.*, 2013).

In primary systemic vasculitides, circulating MP have been previously demonstrated as biomarkers of endothelial injury (Brogan *et al.*, 2004; Clarke *et al.*, 2010). Specifically, EMP have been found to be increased in children (Brogan *et al.*, 2004) and adults (Erdbruegger *et al.*, 2008) with active disease compared to healthy controls. Levels of neutrophil derived MP (NMP) in ANCA-associated vasculitides were elevated in patients with active disease, and levels declined in response to treatment (Daniel *et al.*, 2006; Hong *et al.*, 2012). In patients with Kawasaki disease, EMP are significantly increased compared to healthy controls. EMP levels were even higher in patients with coronary artery aneurysms (Guiducci *et al.*, 2011; Shah *et al.*, 2015) and their levels positively correlated with TNF-α and negatively with albumin , respectively (Tan *et al.*, 2013). Moreover, EMP are involved in cytokine production and progression of vasculitis in the acute phase of Kawasaki disease through specific microRNAs encapsulated in EMP (Nakaoka *et al.*, 2018). Finally, MP have also been studied in

several other autoimmune diseases including Behçet's disease (Khan *et al.*, 2016), inflammatory bowel disease (Voudoukis *et al.*, 2016), diabetes mellitus (Tramontano *et al.*, 2010) and multiple sclerosis (Sheremata *et al.*, 2006).

5.2.5 The role of MP in vascular diseases

Although MP are constitutively secreted in low concentrations in the blood under physiological settings, different types of MP, with EMP playing a crucial role, are implicated in a wide range of diseases involving endothelial injury, activation and vascular damage. For example, circulating EMP are elevated in patients with diabetes mellitus (Jansen *et al.*, 2013). It has been shown that when endothelial cells are cultured under high glucose levels, they release EMP inducing endothelial dysfunction, vascular inflammation and advanced atherosclerosis in vivo (Jansen *et al.*, 2013). Moreover, increased levels of EMP are associated with significant impairment in angiogenesis in vitro as it was observed when endothelial cells were treated with pathophysiological concentrations of EMP (Mezentsev *et al.*, 2005). Other diseases characterised by endothelial dysfunction and injury driven by increased EMP include pre-eclampsia (Petrozella *et al.*, 2012), thrombotic thrombocytopenic purpura (Jimenez *et al.*, 2001), and chronic renal failure (Faure *et al.*, 2006). Apart from EMP, PMP are also increased in patients with chronic renal failure especially in the presence of thrombotic events (Faure *et al.*, 2006).

Altered levels of circulating MP secondary to activation and/or apoptosis of blood and vascular cells have been found in patients with cardiovascular diseases, including acute

coronary syndromes (Bernal-Mizrachi *et al.*, 2003), stroke, aneurysmal arterial disease, venous thromboembolism or peripheral vascular disease (Diamant *et al.*, 2004; Boulanger *et al.*, 2006). As already discussed, MP have strong prothrombotic properties secondary to the high expression of PS and TF in their surface. Stimulation both with TNF-α and IL-1α induces the secretion of TF-rich EMP capable to induce thrombin generation in vivo and in vitro (Combes *et al.*, 1999; Abid Hussein *et al.*, 2008). In support of this, TF-rich MP have been found to be elevated in sickle cell disease, suggesting their possible role in thrombotic events (Shet *et al.*, 2003). It is characteristic that MP also relate to risk factors of cardiovascular events such as hypertension, hyperlipidaemia and obesity (Bakouboula *et al.*, 2008; Yuana *et al.*, 2011). Eleftheriou *et al*. demonstrated increased levels of total circulating MP in children with stroke compared to healthy controls (Eleftheriou *et al.*, 2012). Interestingly, children with recurrent arterial ischaemic stroke had higher numbers of MP compared to those with no recurrence (Eleftheriou *et al.*, 2012).

5.2.6 MP in inflammatory myopathies

Increased numbers of circulating MP are seen in vascular, autoimmune and inflammatory conditions. As IIM represent a heterogeneous group of autoimmune diseases where vasculopathy plays a central role, one could hypothesise that MP could also be increased in that group of diseases, and might contribute to disease pathogenesis, and provide a biomarker of vasculopathy. So far, only a few studies have been performed investigating the presence of MP in adults with dermatomyositis/polymyositis; and no study has examined these in children with JDM. Shirafuji *et al.* demonstrated increased PMP in patients with

DM/PM (Shirafuji *et al.*, 2009); a year later, Baka *et al.* first described increased numbers of monocyte, T- and B-cell derived MP in patient with DM/PM (Baka *et al.*, 2010). Interestingly, although there was no difference between males and females, polymyositis and dermatomyositis, patients with anti-Jo-1 antibodies and lung involvement demonstrated higher number of MP (Baka *et al.*, 2010). When Oyabu *et al.* compared PMP levels between patients with different rheumatic diseases, DM/PM patients had PMP levels similar to the ones observed in SLE patient and patients with mixed connective tissue disease and SSc demonstrated the highest levels (Oyabu *et al.*, 2011).

As JDM has been recognised as a chronic small vessel vasculopathy, it is of interest to investigate whether children with JDM have high levels of MP compared to healthy control, and to explore MP profiling of these patients to look for associations with other indices of endothelial injury. Moreover, MP could exert important prothrombotic effect and might add insight into our understanding of vasculopathic complications which are known to be related with increased disease severity and poorer outcome (Gitiaux *et al.*, 2016).

5.3 Aims

To investigate the MP profiles associated with endothelial activation and cellular activation in children with JDM, and to examine whether MP could serve as a biomarker for monitoring disease activity relating to the vasculopathy of JDM.

5.4 Methods

In the cross-sectional part of this study, children with JDM presenting to GOSH from September 2015 to December 2017 were recruited. Definitions and inclusion/exclusion criteria were discussed in previous sections (2.1.1 and 2.1.4). Healthy controls were recruited as described in section 2.1.2. Patients were also evaluated prospectively at time of recruitment and a follow-up visit. Details of MP identification and enumeration were described in chapter 2, section 2.2.6.

5.5 Results

5.5.1 Circulating MP in children with JDM

MP profile was assessed in 90 patients with JDM. Sixty-four/90 (71.4%) of the patients had active disease as per modified PRINTO criteria. Patients and healthy controls demographic characteristics have been previously described in section 3.4.1 (**Table 3.1**). Total AnV+ MP were higher in patients with JDM median 204.7 (IQR, 87.9-412.6) $x10^3$/ml compared to healthy controls median 44.3 (IQR, 15.0-249.1) $x10^3$/ml, $p<0.0001$ (**Table 5.3)**. Patients with active disease had higher numbers of total AnV+MP median 300.1 (IQR, 186.3-584.6) $x10^3$/ml compared to patients with inactive disease median 81.3 (IQR, 33.8-190.5) $x10^3$/ml, $p<0.0001$ (**Figure 5.1**). Those MP were mainly of platelet and endothelial origin. Interestingly, B-cell derived MP were the third commonest MP population. Comparisons of JDM patients with healthy controls are shown in **Table 5.3.** Total AnV+ MP correlated with CEC levels ($r=0.37$, $p<0.0001$) **(Figure 5.2).**

Microparticle (MP) profile	JDM patients (median, IQR) x 10^3/ml (n = 90)	JDM patients with active disease (median, IQR) x 10^3/ml (n = 64)	JDM patients with inactive disease (median, IQR) x 10^3/ml (n =26)	Healthy Controls (median, IQR) x 10^3/ml
Total AnV+MP	204.7 (87.9-412.6); p<0.0001	300.1 (186.3-584.6); p<0.0001	81.3 (33.8-190.5); p=0.24	44.3 (15.0-249.1)
AnV+CD62E+CD42a-	4.1 (1.1-11.6); p=0.02	6.1 (20.3-2.5); p=0.0001	1.8 (0.3-6.8); p=0.79	2.3 (0.5-5.1)
AnV+CD42a+	77.6 (22.1-161.4); p=0.002	123.1 (50.2-198.3); p<0.0001	28.3 (8.0-47.7); p=0.88	18.1 (6.3-98.3)
TF+CD14+AnV+	0.4 (0.0-2.9); p=0.002	0.6 (0.1-4.3); p=0.0003	0.2 (0.0-1.5); p=0.17	0.1 (0.0-0.6)
CD19+AnV+CD42a-	3.4 (1.2-14.0); p.02	5.2 (1.7-24.6); p=0.0003	1.3 (0.6-5.4); p=0.99	1.8(0.5-6.3)
CD3+AnV+Cd42a-	1.0 (0.2-3.8); p=0.06	1.5 (0.4-5.2);p=0.006	0.6 (0.0-1.5); p=0.98	0.5 (0.1-1.9)

Abbreviations: AnV=Annexin V, JDM=Juvenile Dermatomyositis, MP=microparticles; TF=tissue factor; IQR: interquartile range. The Kruskal-Wallis test followed by the Mann Whitney U test was used to examine differences between study groups. P values reported refer to comparison with the healthy control group. P values of less than 0.05 (2-sided) were regarded as significant.

Table 5.3. Circulating Microparticle profile in Juvenile dermatomyositis (JDM) patients compared to healthy controls. *The Kruskal-Wallis test followed by the Mann Whitney U test was used to examine differences between study groups. Results are presented as median and interquartile range.*

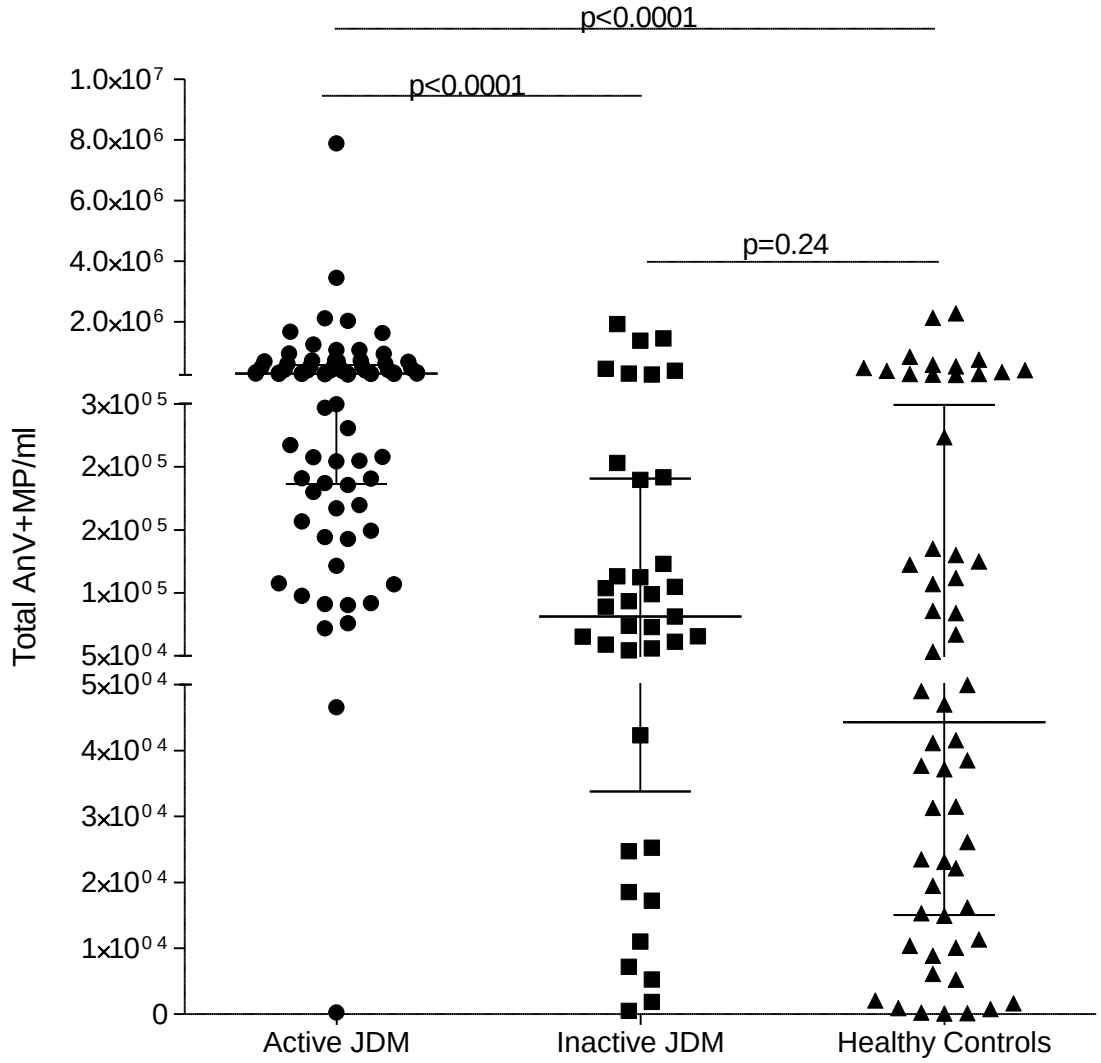

Figure 5.1. Total Annexin V (AnV)+ microparticles in children with Juvenile dermatomyositis (JDM) based on disease activity compared to healthy controls. *Total AnV+ MP in children with active JDM were elevated compared to children with inactive JDM, p<0.0001 and healthy controls, p<0.0001. There was no statistical significant difference in total AnV+ MP between children with inactive disease and healthy controls, p=0.24. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

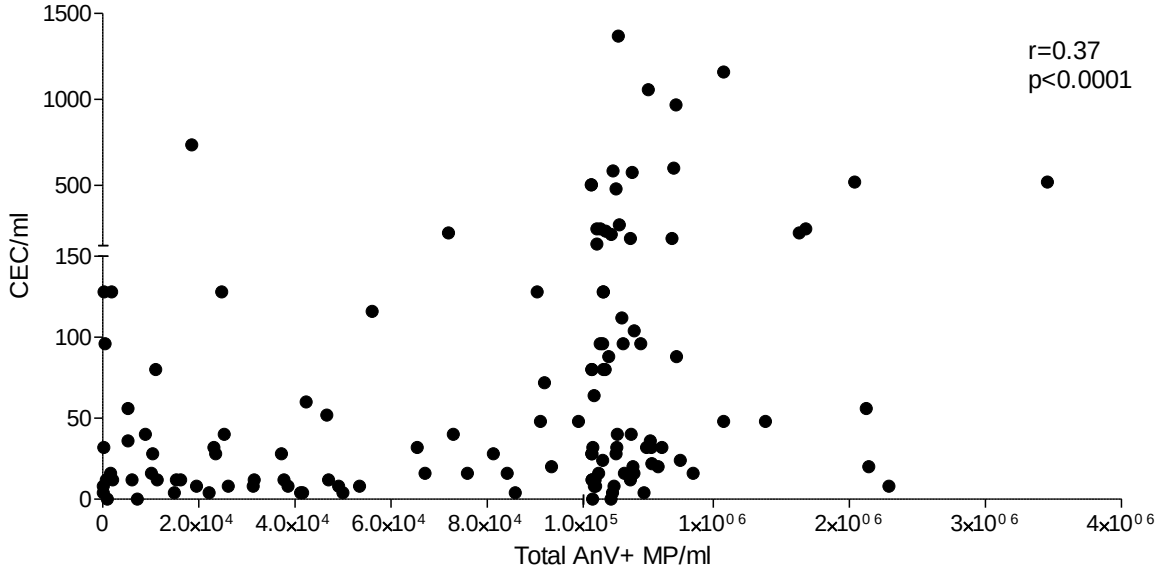

Figure 5.2. Correlation of total Annexin V (AnV)+ microparticles (MP) correlated with circulating endothelial cells (CEC). *Total AnV+ MP correlated with CEC, r=0.37, p<0.0001. Correlation was tested with Spearman's correlation coefficient.*

PMP (CD42a+Anv+) were elevated in children with JDM median 77.6 (IQR, 22.1-161.4) x 10^3/ml compared to healthy controls median 18.1 (IQR, 6.3-98.3) x 10^3/ml, p=0·002. Children with active JDM had increased PMP median 123.1 (IQR, 50.2-198.3) x 10^3/ml when compared to children with inactive disease median 28.3 (IQR, 8.0-47.7) x 10^3/ml, p<0.0001 and healthy controls median 18.1 (IQR, 6.3-98.3) x 10^3/ml, p<0.0001, but there was no difference between those with inactive JDM and healthy controls, p=0.87 (**Figure 5.3**). In addition, EMP expressing the endothelial activation marker CD62E (E-selectin), but negative for the platelet marker CD42a (CD62E+CD42a-AnV+ EMP) were significantly higher in children with active JDM at a median of 6.1 (IQR, 20.3-2.5) x 10^3/ml compared to children with inactive disease with median

of 1.8 (IQR, 0.3-6.8) x 10^3/ml, p=0.0005 and healthy controls median 2.3 (IQR, 0.5-5.1) x 10^3/ml, p<0.0001 (**Figure 5.4**). Of note, circulating EMP correlated significantly with CEC, r=0.20, p=0.027 suggesting a significant association between both biomarkers of endothelial injury (**Figure 5.5**).

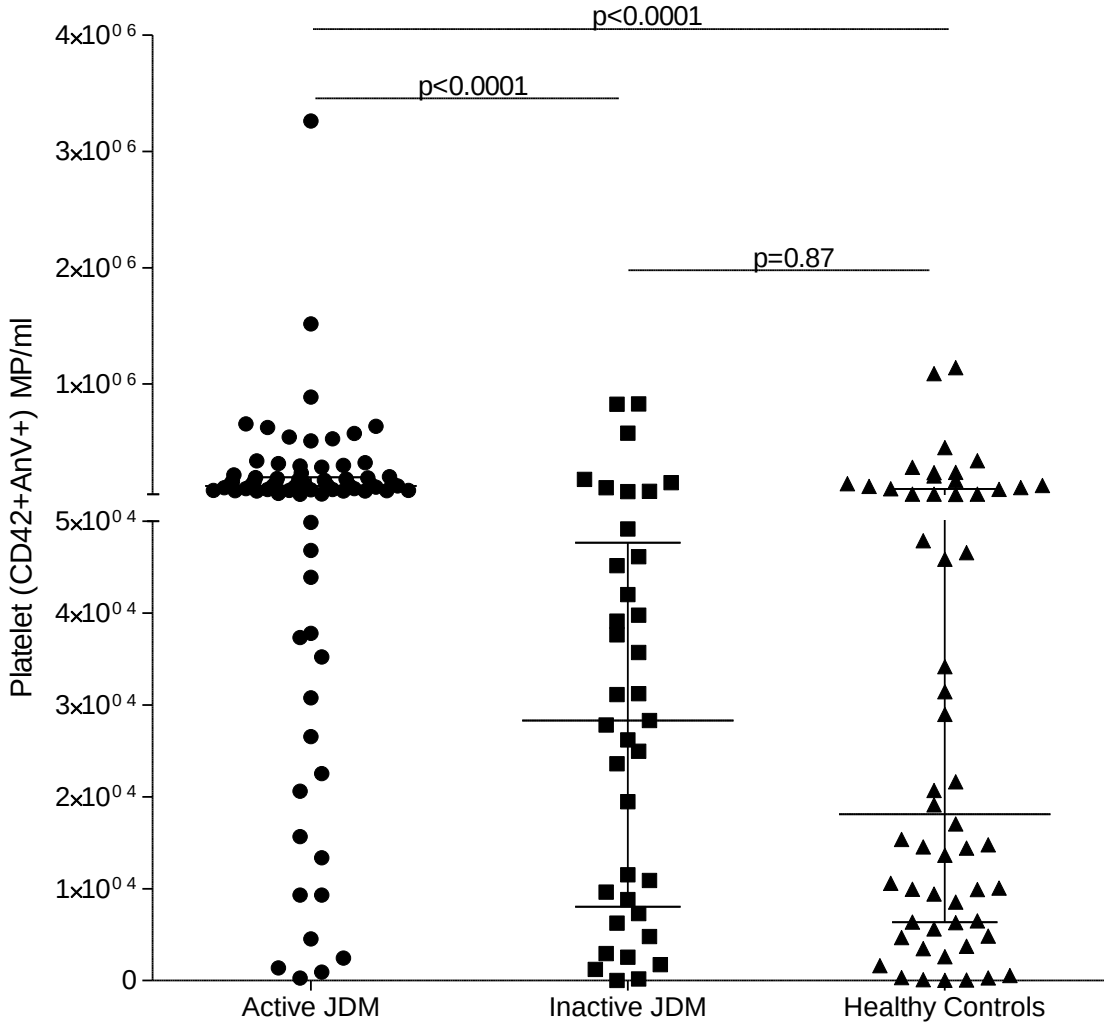

Figure 5.3. Platelet derived MP (PMP) (CD42a+Anv+) in children with Juvenile dermatomyositis (JDM) based on their disease activity and compared to healthy controls.

PMP were elevated in children with JDM median 77.6 (interquartile range, IQR, 22.1-161.4) x

10^3/ml compared to healthy controls median 18.1 (IQR, 6.3-98.3) x 10^3/ml, p=0·002. Children with active JDM had increased PMP median 123.1 (IQR, 50.2-198.3) x 10^3/ml when compared to children with inactive disease median 28.3 (IQR, 8.0-47.7) x 10^3/ml, p<0.0001 and healthy controls median 18.1 (IQR, 6.3-98.3) x 10^3/ml, p<0.0001. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.

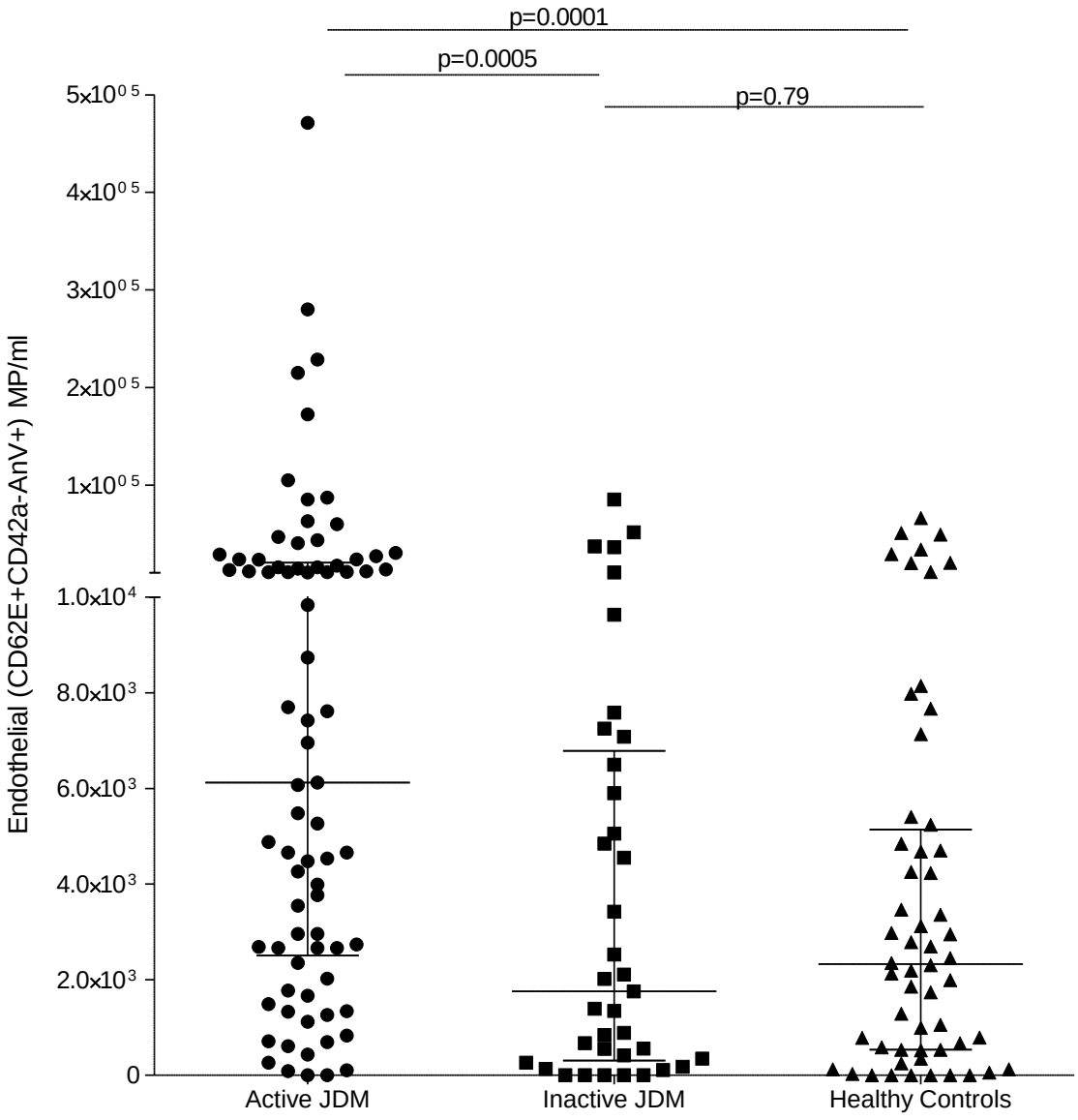

Figure 5.4. Endothelial (CD62E+CD42a-AnV+) microparticles (EMP) in children with Juvenile dermatomyositis (JDM) based on disease activity compared to healthy controls. *EMP in active JDM median 6.1 (interquartile range, IQR, 20.3-2.5) x 10^3/ml were elevated compared to inactive median 1.8 (IQR, 0.3-6.8) x 10^3/m, p=0.0005 and healthy controls median 2.3 (IQR, 0.5-5.1) x 10^3/ml, p<0.0001, but there was no difference between children with inactive*

disease and healthy controls, p=0.79. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.

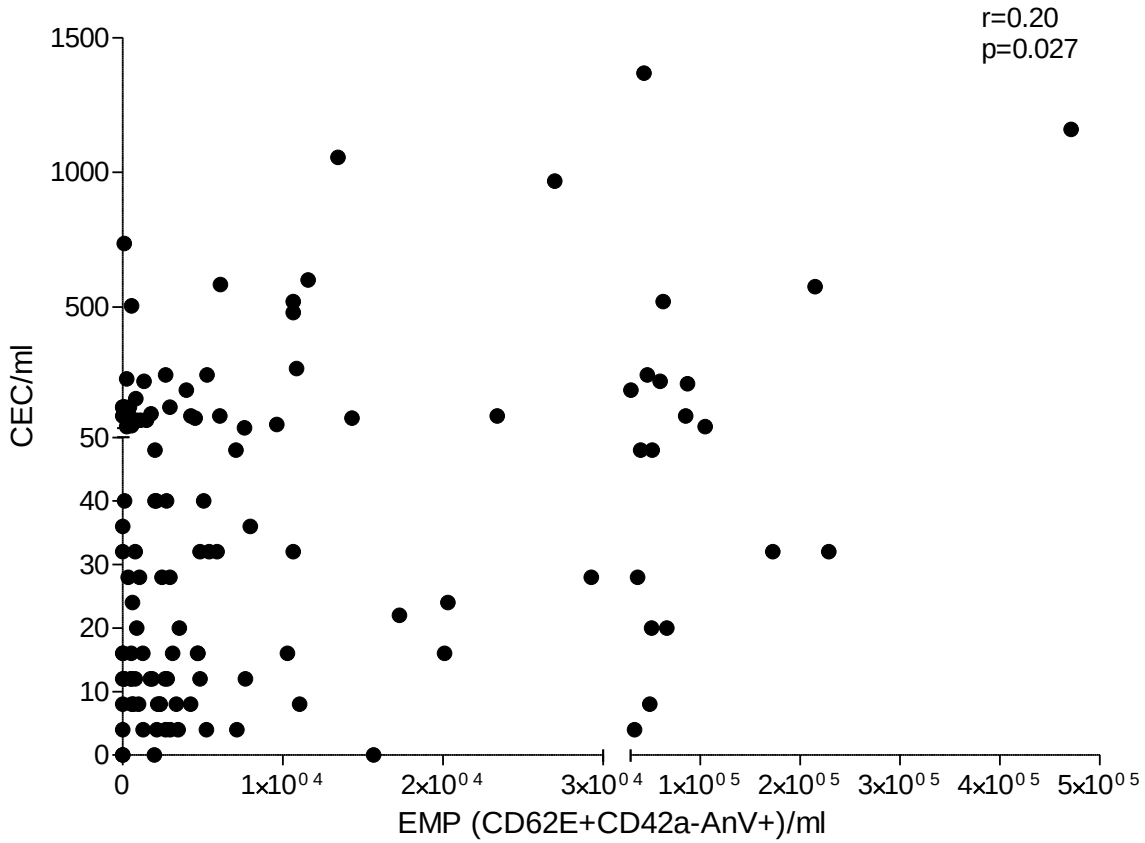

Figure 5.5. Correlation of endothelial (CD62E+CD42a-AnV+) microparticles (EMP) with circulating endothelial cells (CEC). *EMP strongly correlated with CEC, r=0.20, p=0.027 in children with Juvenile Dermatomyositis (JDM) and healthy controls. Correlation calculated with Spearman's correlation coefficient.*

B-cell derived MP defined as CD19+CD42a-AnV+ MP were significantly higher in active JDM patients at a median of 5.2 (IQR, 1.7-24.6) x 10^3/ml compared to patients with inactive disease

median 1.3 (IQR, 0.6-5.4) x 10^3/ml, p=0.002 and healthy controls median 1.8 (IQR, 0.5-6.3) x 10^3/ml, p=0.0003 (**Figure 5.6**). T-cell derived MP defined as CD3+CD42a-AnV+ MP did not differ significantly between children with JDM median 1.0 (IQR, 0.2-3.8) x 10^3/ml and healthy controls median 0.5 (IQR, 0.1-1.9) x 10^3/ml, p=0.06 (**Figure 5.7**). TF expression on MP of monocytic (CD14) origin was then compared between the groups since these are known to be highly prothrombotic. TF+CD14+AnV+ MP were significantly elevated in those with active JDM median 0.6 (IQR, 0.1-4.3) x 10^3/ml when compared to healthy controls median 0.1 (IQR, 0.0-0.6) x 10^3/ml, p=0.0003 but there was no difference when compared to children with inactive disease median 0.2 (IQR, 0.0-1.5) x 10^3/ml, p=0.06 (**Figure 5.8**).

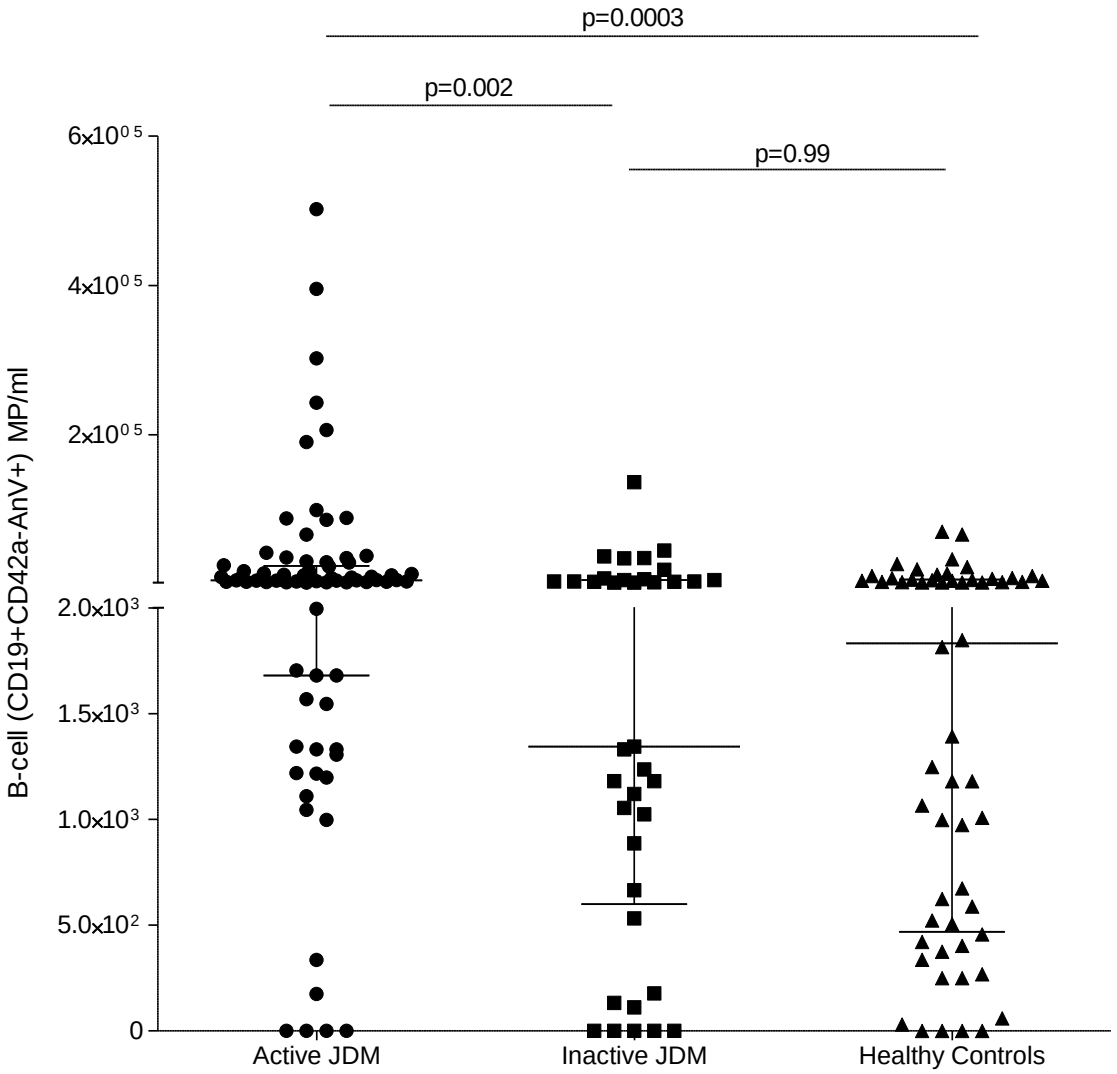

Figure 5.6. B-cell (CD19+CD42a-AnV+) microparticles (MP) in children with Juvenile dermatomyositis (JDM) based on their disease activity compared to healthy controls. *Patients with active JDM had higher numbers of B-cell MP compared to those with inactive disease, p=0.02 and healthy controls, p=0.003. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

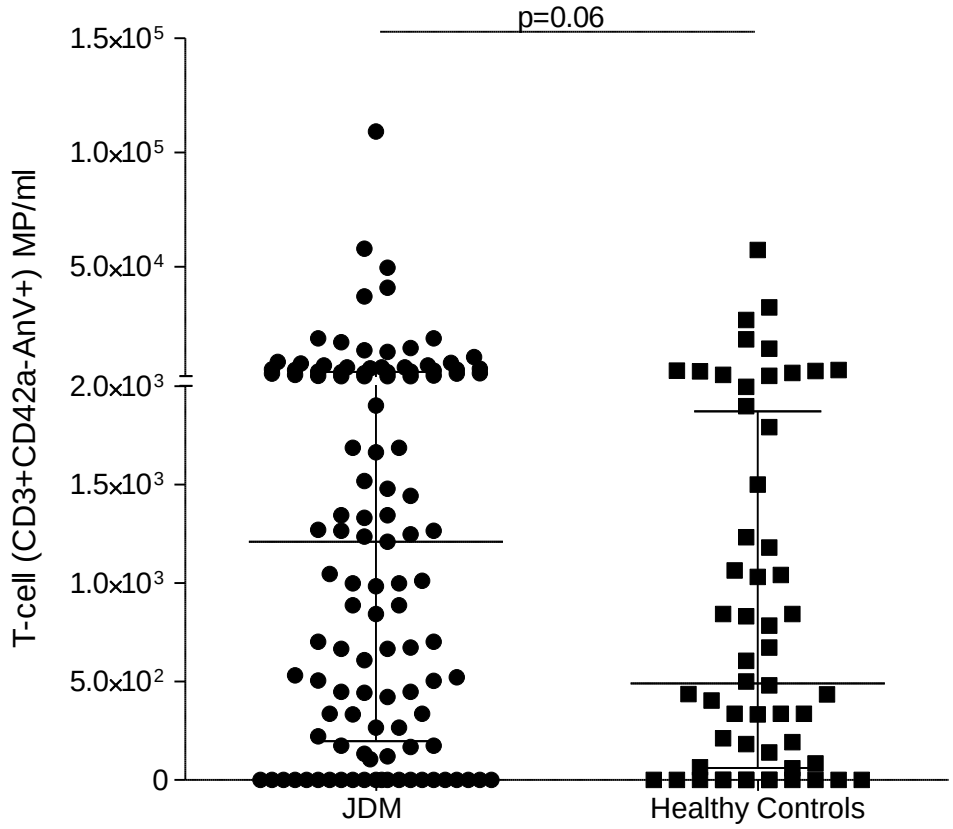

Figure 5.7. T-cell (CD3+CD42a-AnV+) derived microparticles (MP) in children with Juvenile dermatomyositis (JDM) and healthy controls. *There was no difference in T-cell (CD3+CD42a-AnV+) MP between groups of patients with JDM and healthy controls, p=0.06. As no difference was observed, no further subgroup analysis was performed in the JDM group based on disease activity. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

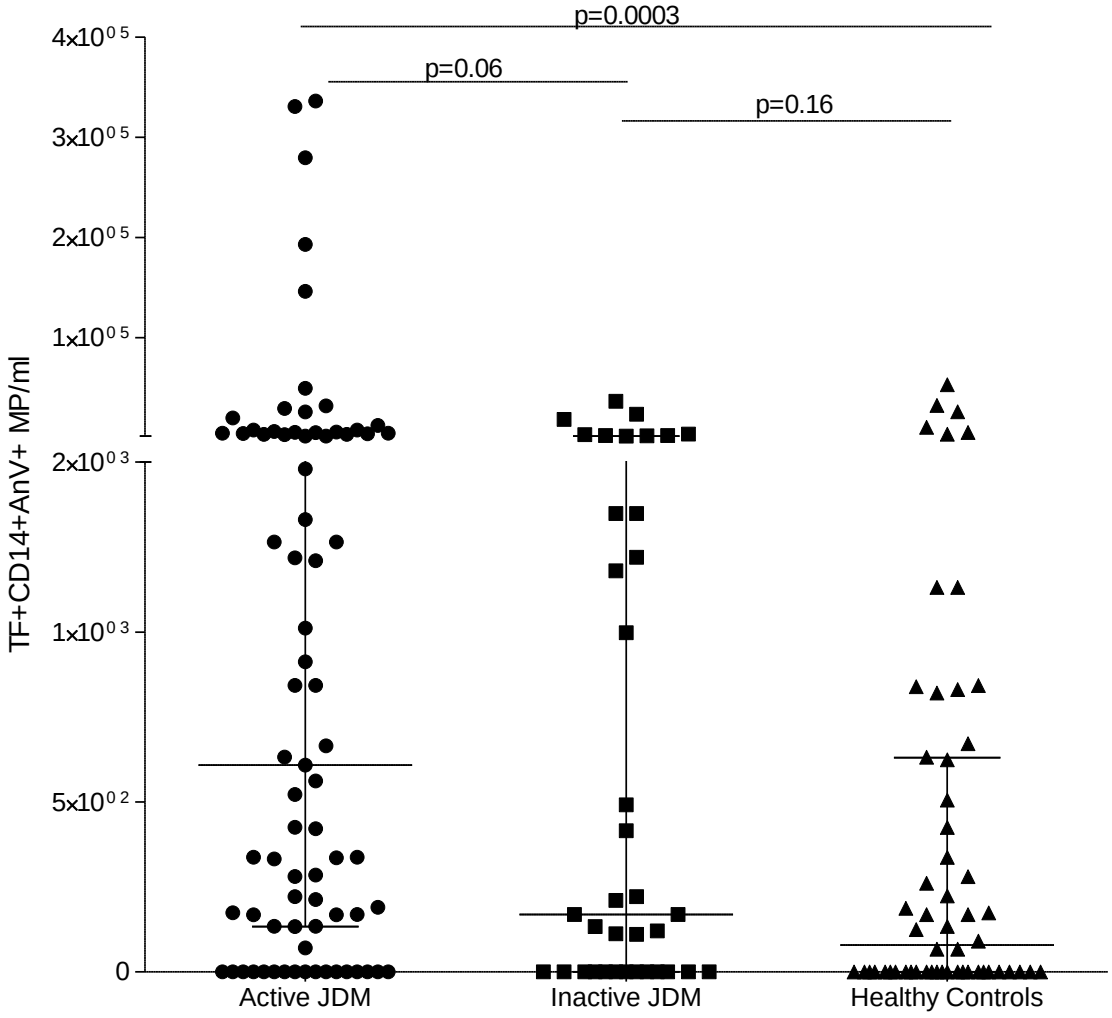

Figure 5.8. Tissue factor (TF)+ monocyte (CD14+) derived microparticles (TF+CD14+AnV+ MP) in children with Juvenile dermatomyositis (JDM) based on disease activity compared to healthy controls. *TF+CD14+AnV+ MP were significantly elevated in children with active JDM median 0.6 (interquartile range, IQR, 0.1-4.3) x 10^3/ml when compared to healthy controls median 0.1 (IQR, 0.0-0.6) x 10^3/ml, p=0.0003 but there was no difference when compared to children with inactive disease median 0.2 (IQR, 0.0-1.5) x 10^3/ml, p=0.06. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

5.5.2 Longitudinal changes of MP in children with JDM

Figure 5.9 summarizes the longitudinal changes for 25 children with JDM that were studied at time 0 (time of recruitment) and during at least one follow-up visit after recruitment. Children with active JDM at time of recruitment that went into remission during the follow-up appointment (n=5) had reduced total AnV+MP at latest follow up with a drop from median 729 (IQR, 297-1556) x 10^3/ml at recruitment to median 105 (IQR, 58-230) x 10^3/ml at last follow-up, p=0.09 (**Figure 5.9A**). Similarly, children with inactive disease at time of recruitment that were found to have active disease at last follow-up visit (n=3) had an increase in total AnV+ MP levels from median 98 (IQR, 65-247) x 10^3/ml at time of recruitment to median 342 (IQR, 121-453) x 10^3/ml at last follow-up visit, p=0.27 (**Figure 5.9B**). In both cases, the differences between the two timepoints did not reach statistical significance, probably due to the small numbers. When all patients that had a change in disease status (from active to inactive or inactive to active JDM) (n=8) were included, the difference in total AnV+ MP almost reached statistical significance, p=0.057 (**Figure 5.9C**).

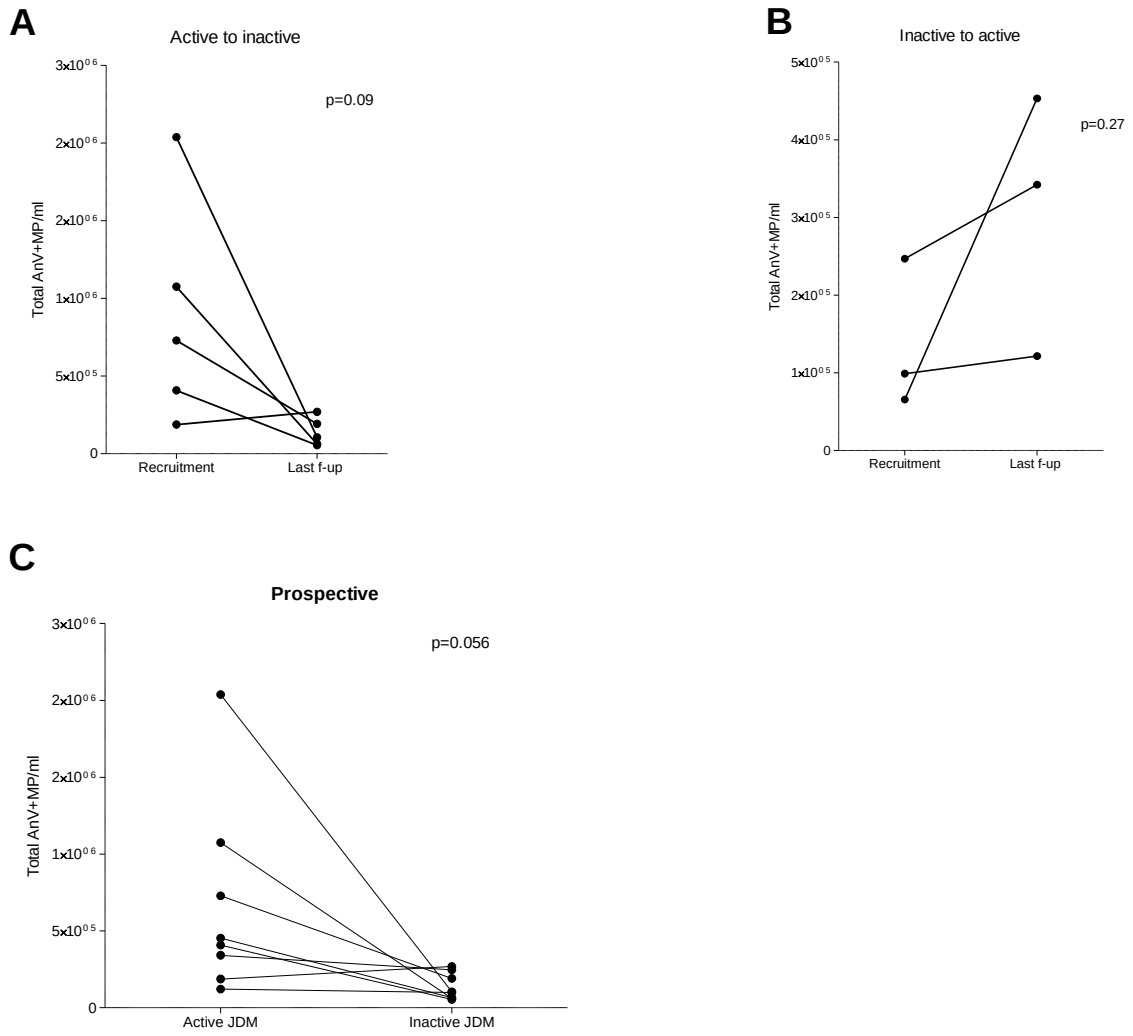

Figure 5.9. Longitudinal changes in total Annexin V + microparticles (AnV+MP) in children with Juvenile dermatomyositis (JDM). *For children with JDM who went into remission at latest follow-up visit (n=5), levels of AnV+MP declined with change from a median of 729 (interquartile range, IQR, 297-1556) x 10^3/ml at time of initial recruitment to a median of 105 (IQR, 58-230) x 10^3/ml at latest follow-up, p=0.09 (**Figure 5.9A**). Similarly, for children with inactive disease at time of recruitment who had a disease flare at latest follow-up visit (n=3) total AnV+MP levels increased from a median of 98 (IQR, 65-247) x 10^3/ml at time of initial recruitment to a median of 342 (IQR, 121-453) x 10^3/ml at latest follow-up visit, p=0.27 (**Figure***

***5.9B**). When all patients were considered (n=8), the difference in total AnV+MP almost reached statistical significance, p=0.057 (**Figure 5.9C**). P values were calculated with Wilcoxon matched pairs signed rank test.*

5.5.3 Test characteristics of MP for identification of children with active JDM

On the basis that total AnV+ MP differed significantly between children with active disease and children with inactive disease, the test characteristics of MP for identification of children with active JDM were then examined by plotting ROC curves. The ROC curve for MP at varying definitions of positivity is shown in **Figure 5.10**. ROC analysis for MP as a biomarker for disease activity was significant with an AUC of 0.81, Standard Error (SE) 0.05 and 95% CI of 0.71-0.91, $p<0.0001$. The full test characteristics of MP for identification of children with active JDM are summarized in **Appendix 5**. The cut-off values for test positivity in **Appendix 5** correspond to individual points on the ROC curves.

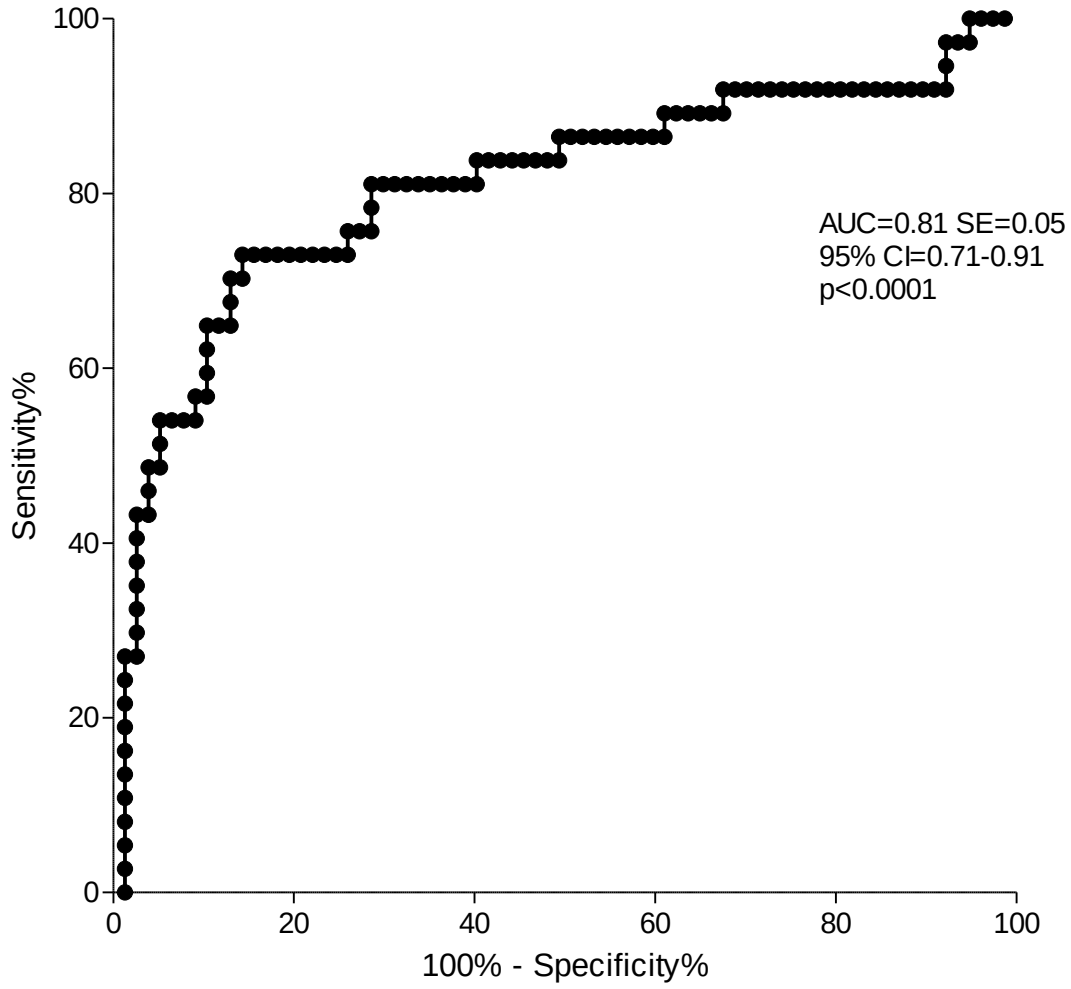

Figure 5.10. Receiver operator characteristic curve (ROC) for total Annexin V positive microparticles for the identification of children with active Juvenile dermatomyositis (JDM).

ROC analysis was significant with an area under the curve (AUC) of 0.81 (95% Confidence Interval 0.71-0.91), p<0.0001.

5.6 Discussion

Children with JDM had higher plasma levels of total AnV+ MP compared to healthy controls. When I examined the detailed circulating MP profile, I was able to show that the MP were mainly of platelet (expressing CD42a), activated endothelial (expressing CD62E) and B-cell origin, while there was no different between T-cell (expressing CD3) derived MP. Notably, monocytic MPs expressing tissue factor were significantly raised in the circulation of children with JDM suggesting an increased pro-thrombotic risk. Moreover, when children with active disease were compared to children with inactive disease, I demonstrated that circulating MP effectively differentiated children with different disease status and may thus provide useful insights into endothelial, platelet, monocyte and B-cell activation in JDM pathogenesis. Interestingly, at the prospective part of the study where 25 JDM patients were studied longitudinally, circulating MP levels tended to change when there was a change in disease activity status suggesting for the first time that MP could serve as a biomarker for monitoring disease activity or predicting disease flares in children with JDM.

The release of EMP reflect one of the primary responses of the damaged or activated endothelium highlighting their role as an early biomarker of vascular dysfunction (Curtis *et al.*, 2013). Moreover, EMP may also represent a biomarker that can help with prognosis of outcomes potential as demonstrated in patients with acute ischaemic stroke (Simak *et al.*, 2004), pulmonary hypertension (Amabile *et al.*, 2009) and chronic renal failure (Forest *et al.*, 2010) where EMP were found to predict disease outcome. EMP are also strongly linked to cardiovascular disease as evidence from studies in patient with acute coronary syndromes

(Mallat *et al.*, 2000), diabetes mellitus (Koga *et al.*, 2005) and SLE (Pericleous *et al.*, 2013) highlighting the fact that EMP do not only serve as a marker of endothelial dysfunction but also play a significant role in inflammatory responses, coagulation, thrombosis but also angiogenesis (Mezentsev *et al.*, 2005; Jung *et al.*, 2015; Yu *et al.*, 2017). In SLE patients, a prototypic connective tissue disease, EMP levels correlated with active disease and impaired brachial artery flow-mediated dilatation (FMD), a marker of endothelial dysfunction, while, interestingly, aggressive immunosuppressive treatment resulting in better disease control, led to a decrease in EMP levels (Parker *et al.*, 2014). Similarly, in this cohort of children with JDM, EMP expressing the surface marker CD62E were significantly elevated in children with active disease compared to those with inactive disease and healthy controls. Moreover, EMP levels were correlated with another marker of endothelial dysfunction, CEC, suggesting that both these markers allow the detection and monitoring of endothelial dysfunction in vivo, although a direct link between those two markers have not yet identified. Finally, it is well described that high numbers of EMP suppress angiogenesis (Mezentsev *et al.*, 2005) suggesting another possible mechanism of EMP contribution to JDM pathogenesis through impaired angiogenesis.

PMP represent the biggest MP subpopulation in human plasma (Berckmans *et al.*, 2001; Joop *et al.*, 2001). Several studies have shown that PMP are implicated in several biological processes including thrombosis (Mezouar *et al.*, 2014), inflammation (Williams *et al.*, 2014), coagulation (Toth *et al.*, 2008) and intercellular communication and interactions (Kim *et al.*, 2004). In this study, PMP were found to be elevated in children with active JDM compared to

those with inactive disease and healthy controls. In keeping with published literature, PMP represent the largest MP subpopulation. Only two studies have previously looked into the levels of PMP in adults with DM/PM with conflicting results. In 2009, Shirafuji *et al*. also demonstrated increased levels of PMP in adults with active disease (Shirafuji *et al.*, 2009), while Oyabu *et al*. did not find any difference between adults with PM/DM and healthy controls (Oyabu *et al.*, 2011). In this cohort, circulating PMP levels did not differ between children with inactive disease compared to healthy controls in contrast with Shirafuji *et al*. where adult patients on treatment had elevated PMP in comparison to healthy controls (Shirafuji *et al.*, 2009). It is important however to highlight that Shirafuji *et al*. only looked in the muscular component of the disease with no reference to skin disease (thus those patients may still have had active disease and thus higher numbers of circulating PMP). There are a number of mechanisms explaining the role of PMP in the pathogenesis of autoimmune diseases which could also apply to JDM. PMP have been suggested to promote endothelial dysfunction through intercellular interaction by delivering miRNA that subsequently upregulates or downregulates gene expression in endothelial cells (Laffont *et al.*, 2013). Similarly, PMP alter monocyte macrophage function toward a more pro-inflammatory and phagocytic phenotype (Laffont *et al.*, 2013). Additionally, it has been previously described that PMP carry mitochondrial DNA (Boudreau *et al.*, 2014) which is known to strongly induce neutrophil extracellular traps (NET) formulation (NETosis) promoting IFN production (Zhang *et al.*, 2010; Oka *et al.*, 2012).

Interestingly, monocyte derived MP expressing TF were elevated in children with active JDM compared to those with inactive disease and healthy controls suggesting for the first time a possible role of MP in pathogenesis of the occlusive vasculopathy demonstrated in children with JDM. Baka *et al.* found that plasma level of monocytic MP were elevated in adults with PM/DM and strongly correlated with MMT but the subgroup of TF expressing monocytic MP was not investigated (Baka *et al.*, 2010). Monocytic MP contribute to the coagulation cascade through the expression of TF and PS (Key and Kwaan, 2010). TF expression on monocytic MP results in thrombin generation (Del Conde *et al.*, 2005). Moreover, TNFα- and IL-1α modulate TF expression on monocytes and TF expressing monocytic MP contain TNFα- and IL-1α TF creating a positive feedback loop that promotes thrombosis (Khan *et al.*, 2010). These observations suggest a possible pathogenetic mechanism of prothrombotic propensity of children with JDM that will be further analysed in chapter 6.

Finally, B-cell and T-cell derived MP were examined in children with JDM in this cohort. B-cell derived but not T-cell derived MP were found to be increased in children to JDM compared to healthy controls. This is partially in keeping with one single previous study that examined the role of B- and T-cell derived MP in adults with DM/PM in which both B- and T-cell derived MP were increased in adults with DM/PM (Baka *et al.*, 2010). Both B-cell and T-cells are implicated in JDM pathogenesis, but it is not known whether MP derived from those cells have a pathogenetic role, amplify the inflammatory process or represent the result of the process.

On the whole, the test characteristics of circulating MP for prediction of disease activity in children with JDM are promising. Furthermore, circulating MP may be useful in monitoring disease activity over time, predict disease flares and guide physician's decision about further therapeutic management as demonstrated by the small longitudinal study. The preliminary prospective data presented here suggest that MP in children with active disease remain elevated and may contribute to thrombosis and therefore to the severe extramuscular complications of JDM. Examining the changes of MP at several time points during the disease course may provide additional information on future organ involvement and effect of treatment. Future studies may also give insights into the endothelial bed origin of EMP in JDM, to establish whether these MP are released as a result of endothelial activation or ischaemia, or both. Overall however the study of EMP provides a window to endothelial activation status, and represents an innovative non-invasive method of monitoring the vasculopathy of JDM.

This study has several limitations. Multicolour flow cytometry was used for the identification and enumeration of MP. As already discussed in section 5.2.2, flow cytometry is unable to detect the smaller MP, thus the exact number of MP may be underestimated. Moreover, the use of a larger number of surface markers may be able to better characterise the phenotype of MP and give more information regarding the status of the cell origin (activated or apoptotic cell). Some MP subpopulations that may play an important role in disease pathogenesis and progression, like neutrophil derived MP, or skeletal muscle derived MP were also not studied. Of note, in this study only MP that expressed PS and strongly bound to AnV were studied, and

thus AnV negative MP were not included. Proteomic analysis to look into the MP composition and provide a better insight into their functional properties has not been performed as that would require larger volumes of blood samples to be used for MP analysis which is very difficult in research conducted in children. As children included in the current cohort were on a number of different combinations of therapeutic agents, MP analysis accounting for different therapeutic regimes was not able due to the small numbers.

In conclusion, significant differences were observed in circulating endothelial, platelet, monocyte and B-cell derived MP between JDM patients and healthy controls. MP profiling could differentiate patients with active disease from those with inactive disease. These observations further support the findings relating to CEC described in previous sections. The combination of the results from CEC and MP analysis in children with JDM provide strong evidence for ongoing endothelial injury and platelet activation in children with active JDM and may have pathophysiological implications. In chapter 6, the overall functional effect of circulating MP with respects to their procoagulant activity in the vasculopathy of JDM will be examined. In children with systemic vasculitis have recently been shown that occlusive thromboembolic phenomena are associated with enhanced MP mediated thrombin generation (Eleftheriou *et al.*, 2011).

6 Thrombotic propensity in children with JDM

6.1 Summary

Background: Growing evidence suggests that patients with idiopathic inflammatory myopathies may have an increased risk of thromboembolic events in their lifetime. JDM is a systemic vasculopathy and micro and macrovascular involvement often determine the different disease trajectories. In the previous chapter, I have shown that active JDM is characterised by increased circulating MP. These MP are known to be procoagulant. Therefore, they may contribute to an enhanced thrombotic propensity in JDM.

Objectives: To investigate a possible hypercoagulable state in JDM patients by examining a plasma thrombin generation assay.

Methods: Ninety patients recruited to the UK JDM Cohort & Biomarker Study were included; median age 10.21 (IQR, 6.68-13.40) years with median disease duration of 1.63 (IQR, 0.28-4.66) years. Inactive disease was defined as per modified PRINTO criteria: no skin rashes, CK≤150 U/l, CMAS≥ 48/52, MMT8≥78/80, PhyGLOVAS ≤ 0.2/10. MP were identified with multicolour flow cytometry, respectively. A commercially available fluorogenic thrombin generation assay was used and the following parameters were measured: lag time, peak thrombin, velocity index and endogenous thrombin potential/area under the curve.

Results: Children with active JDM exhibited a significantly higher median peak thrombin of 182.1 (IQR, 126.7-270.6) nM compared to the children with inactive JDM, median peak thrombin 128.2 (IQR, 90.4-166.8) nM, $p<0.0001$; and healthy controls, median peak thrombin 44.8 (IQR, 22.8-78.6) nM, $p<0.0001$. Both active and inactive JDM patients had a shorter lag

time when compared to healthy controls, p<0.0001 and p=0.0001, respectively. For children with active JDM, there was in addition a higher median velocity index (p=0.002 and p<0.0001, respectively); and higher median ETP compared to children with inactive JDM and healthy controls (p=0.0004 and p<0.0001, respectively).

Conclusion: Children with active JDM exhibit enhanced thrombin generation compared with children with inactive disease and healthy controls. I therefore demonstrated for the first time that children with JDM are in a chronic prothrombotic state that may contribute to the vascular complications associated with poorer disease outcomes, and may contribute to an increased risk of thromboembolic events later in adulthood.

6.2 Introduction

There is accumulating evidence that patients with chronic inflammatory diseases have an increased risk of cardiovascular disease including coronary heart disease, stroke, peripheral artery disease and venous thromboembolism (Dregan *et al.*, 2014; Mason and Libby, 2015). There has been a recent interest in studying the risk of venous thrombotic events (VTE), mainly deep vein thrombosis (DVT) and pulmonary embolism (PEM), in patients with DM/PM (Selva-O'Callaghan *et al.*, 2011; Nowak *et al.*, 2016). Carruthers *et al*. (Carruthers *et al.*, 2016) demonstrated an increased overall risk of VTE both in patients with PM and DM, 6 times and 8 times respectively (Carruthers *et al.*, 2016). Looking into the different subtypes of VTE, risk of PEM was increased only in PM patients. When considering the VTE risk according to disease onset, the risk was highest during the first year for PM; and the first two years for DM, respectively (Carruthers *et al.*, 2016). In a Swedish study, patients with PM/DM had three

times higher overall risk of PEM compared to the general population, with a 16 times higher risk during the first year after disease diagnosis (Zoller *et al.*, 2012a). Similarly, a UK study found a three times increased risk of VTE in PM/DM patients admitted to hospital (Ramagopalan *et al.*, 2011). Recently, a meta-analysis including 6 studies and 9,045 patients with PM/DM suggested that inflammatory myopathies are associated with an increased risk of VTE (OR=4.31, 95% CI: 2.55–7.29, $P<0.001$) showing strong evidence for the association between inflammatory myopathies and both DVT and PEM (Li *et al.*, 2018).

Despite the strong evidence of thrombotic propensity in patients with inflammatory myopathies, the exact mechanism has not been yet fully defined. Based on Virchow's triad, venous stasis, hypercoagulability and endothelial damage are the main contributors of venous thrombosis (Xu *et al.*, 2010). Patients with inflammatory myopathies are usually characterised by decreased mobility which can subsequently affect venous stasis. Moreover, inflammation, a key feature of inflammatory myopathies, has been associated with a hypercoagulable state through different suggested pathways. TF is the main activator of the extrinsic coagulation pathway. The main cells expressing TF include monocytes, endothelial cells and vascular smooth muscle cells. Factor VIIa forms a complex with exposed TF resulting in further activation of the coagulation cascade (Spronk *et al.*, 2003). The complex of TF-VIIa further activates factor X which then binds to factor Va. The complex Va-Xa then converts prothrombin to thrombin (Esmon, 2006). Two pathways are mainly implicated in the exposure of TF. The first one includes the exposure of TF through interruption of the endothelial layer secondary to the vascular injury (Tomasson *et al.*, 2009) seen in several inflammatory

disorders. The second one is associated with the role of several inflammatory cytokines in the increased expression of TF on endothelial cells and monocytes. For example, IL-6 has been reported to increase the expression of TF in monocytes (Kerr *et al.*, 2001). Inflammation also contributes to hypercoagulability through the reduction of several anticoagulant factors. Both IL-1 (Nawroth *et al.*, 1986; Archipoff *et al.*, 1991)and TNF-α have been shown to decrease TM levels by blocking its transcription (Conway and Rosenberg, 1988; Murray *et al.*, 1991). TM which is mainly expressed on endothelial cells has an anticoagulant effect by binding thrombin and preventing thrombin's effect on clot formulation. Finally, inflammation is linked with coagulation through the fibrinolytic system. Plasminogen is converted to plasmin through two main types of plasminogen activators, the tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), the activity of which are regulated by the plasminogen activator inhibitor (PAI) type-1 and PAI-2. Plasmin is then converting fibrin to soluble fibrin degradation products (FDP), the activity of which is controlled by α2-antiplasminogen (Medcalf, 2007). Of note, thrombin activates thrombin-activatable fibrinolysis inhibitor, which inhibits fibrinolysis showing an interaction between the coagulation and fibrinolysis pathway (Medcalf, 2007; Rijken and Lijnen, 2009). TNF-α has been demonstrated to markedly suppress fibrinolysis (Agirbasli *et al.*, 2006; Medcalf, 2007) which in combination with the fact that fibrinogen -/- mice had reduced macrophage adhesion and decreased production of cytokines during inflammation (Szaba and Smiley, 2002) clearly demonstrate the strong interconnections between inflammation and fibrinolysis.

A recent study by Silverberg *et al*. reported increased associations between JDM and peripheral atherosclerosis, transient ischaemic attacks and cerebral infarction, late effects of cerebrovascular disease and pulmonary circulatory disorder even in the absence of obesity, hypertension and diabetes (Silverberg *et al.*, 2018) with most of these disease complications happening in adolescence. In keeping with these findings, several smaller studies have demonstrated that children with JDM have increased cardiovascular risk factors including metabolic abnormalities (Misra *et al.*, 2004; Coyle *et al.*, 2009) and higher rates of systolic and diastolic cardiac dysfunction when compared to healthy controls (Schwartz *et al.*, 2014; Barth *et al.*, 2016). In contrast to the several studies in adult patients with DM/PM, little is known about coagulation abnormalities and associated comorbidities in children and adolescents with JDM. The lack of evidence in combination with the lack of clear understanding of the causative factors of these life-threatening disease complications, has led to the complete absence of biomarkers able to identify patients at increased risk and total lack of guidance regarding the need of thrombosis prevention policies in children and adolescents with JDM. Children with JDM are characterized by a relatively short disease course without co-existing vascular complications, and the absence of possible confounders like long-term smoking, alcohol or drug abuse. This allows the investigation of the influence of the disease itself on haemostasis without bias.

6.2.1 Thrombin generation assays

Thrombin is the end product of a number of catalytic reactions. It has a key role in the conversion of fibrinogen to fibrin and in the activation of procoagulant factors V, VIII, IX and

XIII. Moreover, when binding to TM, thrombin activates the anticoagulant zymogen protein C. Thrombin can also cause platelet activation, can regulate endothelial cell function which in combination with the direct effect on a numerous cell types highlights its importance in haemostasis. Coagulation cascade starts when TF, from the disrupted endothelium, activates FVII forming a complex (van Veen *et al.*, 2008) which then results in the formulation of small amount of activated factors IX and X (Girard *et al.*, 1990; Gailani and Broze, 1991). Activated factor Xa then triggers the generation of thrombin which in turn triggers factor IX, VIII and V activation. At this stage the complex VIIIa-IXa triggers the generation of a significant quantity of Xa sufficient to form a complex of factors called prothrombinase complex which consists of factors Va, Xa, Ca^{+2} and phospholipids. The prothrombinase complex then generates an explosive production of thrombin (Mann *et al.*, 2003). Thrombin then has two paradoxically different functions (Griffin, 1995). From one point of view, thrombin has strong procoagulant properties by converting fibrinogen to a solid fibrin clot. Phosphatidylserine surfaces are now known to be essential in facilitating this process (Hoffman and Monroe, 2001). Conversely, thrombin has also anticoagulant activities by binding to thrombomodulin, an endothelial cell receptor causing activation of protein C (Esmon, 2003). Activated protein C then inactivates factors Va and VIIIa (Esmon, 2003) downregulating the coagulation cascade. Thrombin is further inhibited by antithrombin (Olson and Chuang, 2002), heparin cofactor II (Tollefsen, 2007) and by the tissue factor pathway inhibitor (Bajaj *et al.*, 2001). Because of its dual role, thrombin has been highlighted as the key mediator of the coagulation cascade. A schematic presentation of the coagulation cascade is presented in **Figure 6.1**.

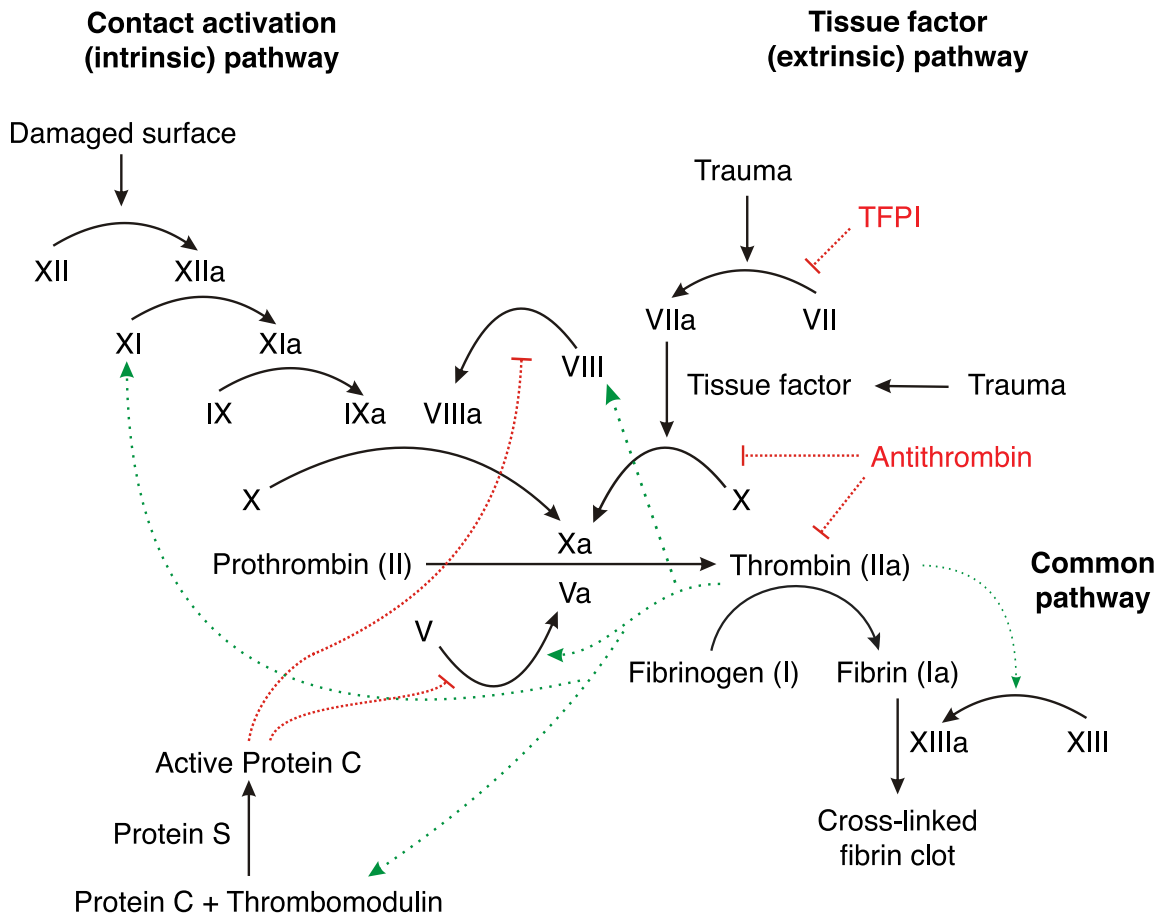

Figure 6.1. The coagulation cascade. *Positive feedback is denoted by green arrows, negative feedback by red arrows. Figure adapted from Knesek et al. (Knesek et al., 2012).*

Traditionally, coagulation state has been investigated with rather basic coagulation tests, including prothrombin time (PT) and activated partial thromboplastin time (APTT). These tests, however, are mainly useful in clinical practice to determine coagulation deficits, and do not accurately detect prothrombotic states. This can be explained firstly by the fact that only 5% of the entire thrombin potential is generated when plasma clots and thus 95% of generated thrombin is disregarded by those tests (Mann, 2003). Secondly, plasma and

reagents used in the measurement of PT and APTT are poor in thrombomodulin and thus the effect of the anticoagulant factors is not properly evaluated. Thrombin generation assays have been created to fill that gap and to better assess the overall effect of procoagulant and anticoagulant factors compared to the conventional coagulation tests (Hemker *et al.*, 2004; van Veen *et al.*, 2008). Thrombin generation assay (TGA) was firstly used back in the 1950s (Macfarlane and Biggs, 1953). In the first used assay, TF or cephaline in combination with calcium chloride were added in whole blood or plasma to trigger the coagulation cascade. At regular intervals, samples of the created mixture were added to a tube containing fibrinogen in order to measure thrombin generation. Thrombin activity was then calculated through a dose-response calibration curve created by the relevant clotting times. The basic principle remained the same until 1986 when Hemker replaced fibrinogen with a chromogenic substrate with affinity to thrombin and used specific computer software to calculate the parameters of the thrombin generation curve (Hemker *et al.*, 1986) which was subsequently called a thrombogram. A few years later, the TGA was further adapted by the use of a synthetic substrate characterised by slower reactivity with thrombin (Hemker *et al.*, 1993). This change was a significant step forward as interval subsampling was no longer required, and the use of automated analysers became feasible. In 2000, the chromogenic substrate was substituted by a fluorogenic substrate whose signal was not negatively affected by the turbidity of the clotting plasma, which was an obstacle with the chromogenic substrate (Hemker *et al.*, 2000). This modification of the TGA has facilitated additionally measurements in platelet rich plasma (Hemker *et al.*, 2000). The most commonly used fluorogenic substrate

is Z-Gly-Gly-Arg-AMC (Hemker *et al.*, 2000). As thrombin cleaves the fluorogenic substrate, it emits fluorescence (Hemker *et al.*, 2000).

Currently, there are three commercially available TGA: two of them using a fluorogenic substrate with the third one using a chromogenic substrate (Kintigh *et al.*, 2018). Overall, the three assays are similar in that all three measure thrombin generation based on the production of a signal through the cleavage of a substrate. Apart from the different substrates used, the major differences include, the sample preparation, and the mathematical modelling used to analyse the data (Kintigh *et al.*, 2018). The most important parameters of a thrombogram are: a) the lag time (min) which corresponds to the time needed for the initiation of thrombin generation; b) the peak thrombin (nM); c) the time to peak thrombin (min); d) the velocity index , which is defined by [peak thrombin/(time to peak thrombin-lag time)]; and e) the area under the curve which corresponds to the endogenous thrombin potential (nM thrombin x min) (**Figure 2.4**). Each one of these parameters has its own significance. Briefly, the lag time represents the clotting time in a traditional clotting test; the peak thrombin represents the highest quantity of thrombin that can be generated; the time to peak thrombin represents the velocity with which thrombin can be generated suggestive of hyper- or hypo-coagulability, respectively; the velocity index is a complex index combining the lag time, the peak thrombin and time to peak thrombin; finally the endogenous thrombin potential represents the total amount of thrombin that can be formulated from the analysed plasma sample (Tripodi, 2016).

To summarise, the TGA has been suggested as a potential assay to fill the current gap between in vivo and ex vivo haemostasis (Tripodi, 2014). It has been proven useful for the study of many diseases including arterial disease and thromboembolic events (Hron *et al.*, 2006; Tripodi *et al.*, 2008; Tripodi, 2016). Autoimmune diseases have been suggested to be associated with an increased risk of cardiovascular diseases. In these diseases, plasma hypercoagulability has been suggested as a possible contributing factor but never validated by clinically available laboratory tests. TGA can detect distinct biomechanical signs of hypercoagulability (Saibeni *et al.*, 2010; Tripodi *et al.*, 2011; Solfietti *et al.*, 2016) and can thus shed a light into mechanisms of coagulation previously poorly understood. In support of this, TGA has been successfully used for the assessment of hypercoagulability in a number of different diseases, such as RA (Solfietti *et al.*, 2016), SLE (Mehta *et al.*, 2010), acute heart failure (Popovic *et al.*, 2019), hyperthyroidism (Kim *et al.*, 2017a), multiple sclerosis (Parsons *et al.*, 2017) and cancer (Ay *et al.*, 2011) Although, JDM is characterised by micro- and macrovascular complications, little is known about coagulation abnormalities in children and adolescents with JDM. TGA could thus be a useful tool for detecting hypercoagulability in children with JDM, and may give an insight into the demonstrated increased risk of thrombotic events in these patients.

6.3 Aims

The aim of this study was to investigate a possible hypercoagulable state in JDM patients by performing a global function test of coagulation – the TGA.

6.4 Methods

6.4.1 Patients and healthy controls

As already described in section 2.1.1, children with JDM recruited through GOSH from September 2015 to December 2017 were studied cross-sectionally. Patients were classified as having inactive disease as per modified PRINTO criteria, section 2.1.4. Recruitment of healthy controls is described in section 2.1.2. Patients were also evaluated prospectively at time of recruitment and a follow-up visit in the prospective part of this study.

6.4.2 Thrombin generation assay (TGA) in plasma

The TGA measures thrombin activity from re-calcified citrated PPP (Bidot *et al.*, 2008; Eleftheriou *et al.*, 2011). 200µL citrated PPP was thawed. 40µL PPP was incubated with 50µL fluorogenic substrate (0.5 mM Z-G-G-R-AMC/7.5 mM Ca^{2+}) and reaction monitored by excitation/emission (360/460nm) at 1min intervals for 90min (Optima Fluorescence plate reader; BMG) as described in section 2.2.7. Lag time, peak thrombin (nM), peak time, velocity index and ETP-area under the curve were quantified according to manufacturer's protocol (Technothrombin®).

6.5 Results

6.5.1 Thrombin generation profile in children with JDM

Thrombin generation profile was assessed in 90 patients with JDM and compared with 81 healthy controls. There were 64/90 patients with active JDM and 26/90 with inactive disease as per modified PRINTO criteria. Patients and healthy controls demographic characteristics have been previously described in section 3.4.1 (**Table 3.1**). None of the included patients and healthy controls were on any anticoagulant treatment at the time of recruitment. Children with active JDM exhibited a significantly higher peak thrombin median 182.1 (IQR, 126.7-270.6) nM compared to the children with inactive JDM, median peak thrombin 128.2 (IQR, 90.4-166.8) nM, p<0.0001; and healthy controls, peak thrombin 44.8 (IQR, 22.8-78.6) nM, p<0.0001; **Figure 6.2**. Children with active JDM had shorter lag time when compared with children with inactive disease, median time 20.0 (IQR, 15.1-28.1) mins and 26.0 (IQR, 15.5-32.7) mins respectively but the difference did not reach statistical significance, p=0.07; **Figure 6.3**. Both active and inactive JDM patients had shorter lag time when compared to healthy controls that had a median time 35.0 (IQR, 24.9-51.0) mins, p<0.0001 and p=0.0001 respectively; **Figure 6.3**. For children with active JDM, there was in addition a higher velocity index median 23.6 (IQR, 12.6-37.7) nM/min; and higher median ETP of 3540 (IQR, 2862-4154) nM x min compared to children with inactive JDM with velocity index median 13.9 (IQR, 9.1-22.7) nM/min, p=0.002; median ETP 2904 (IQR, 2175-3480) nM x min, p=0.0004; and healthy controls with median velocity index 2.8 (IQR, 0.6-7.2) nM/min, p<0.0001, **Figure 6.4**; median ETP 1000 (IQR, 387-1945) nM x min, p<0.0001, **Figure 6.5**.

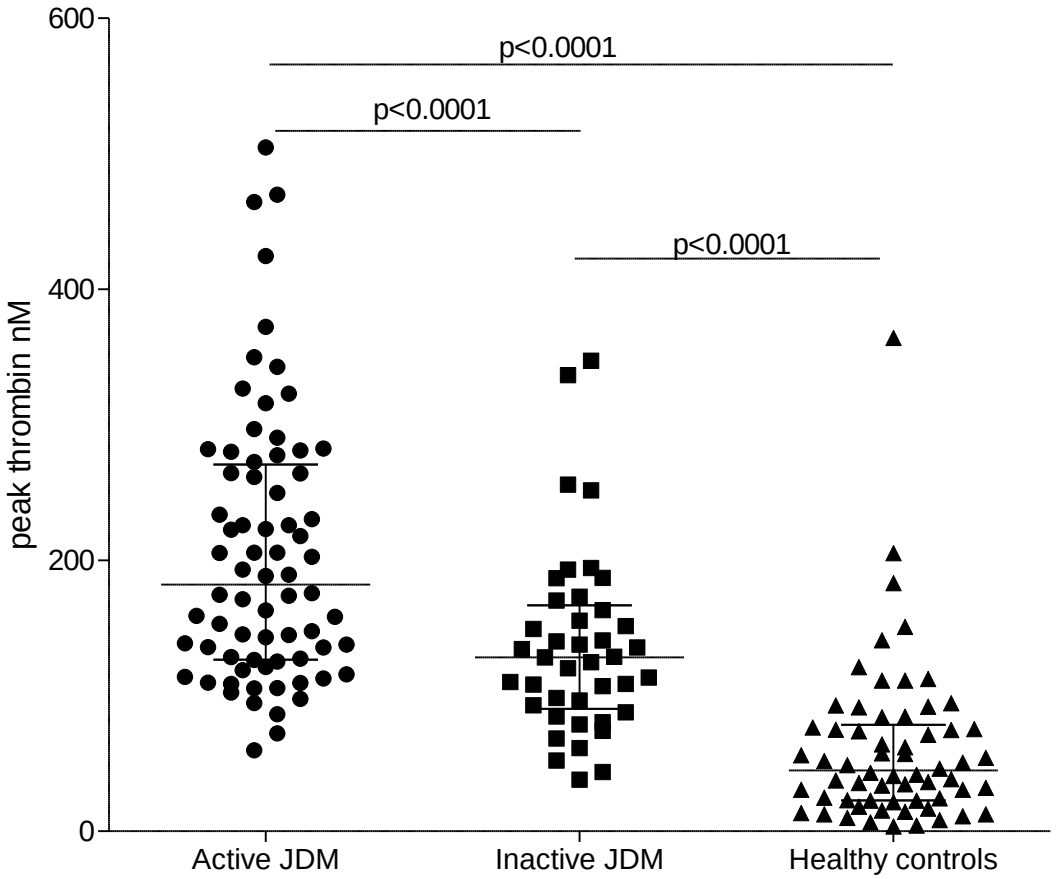

Figure 6.2. Peak plasma thrombin in children with Juvenile dermatomyositis (JDM). *Children with active JDM exhibited a significantly higher peak thrombin compared to the children with inactive JDM, p<0.0001; and healthy controls, p<0.0001. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

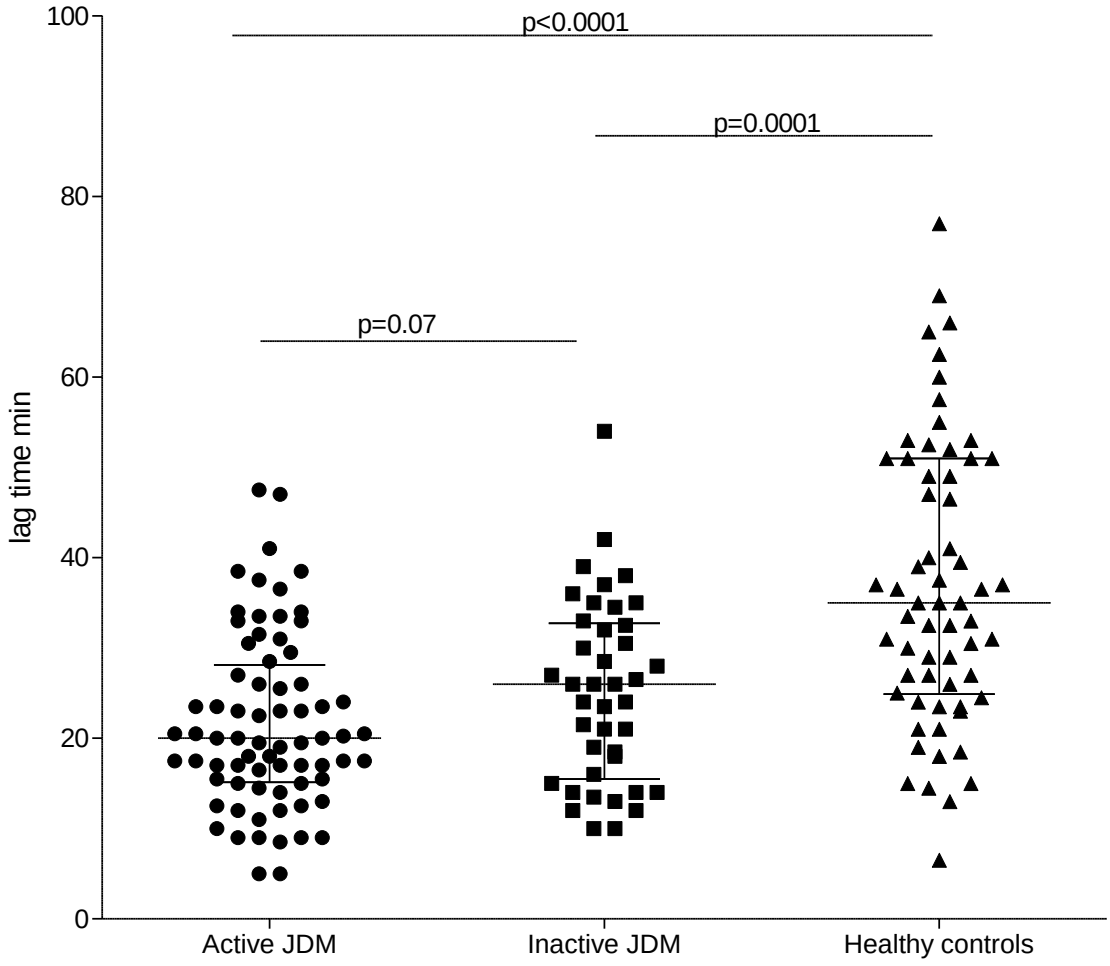

Figure 6.3. Plasma thrombin generation assay lag time (min) in children with Juvenile dermatomyositis (JDM). *Children with active JDM had shorter lag time compared to children with inactive disease, p=0.07. Both children with active and inactive disease had a shorter lag time when compared to healthy controls, p<0.0001 and p=0.0001, respectively. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

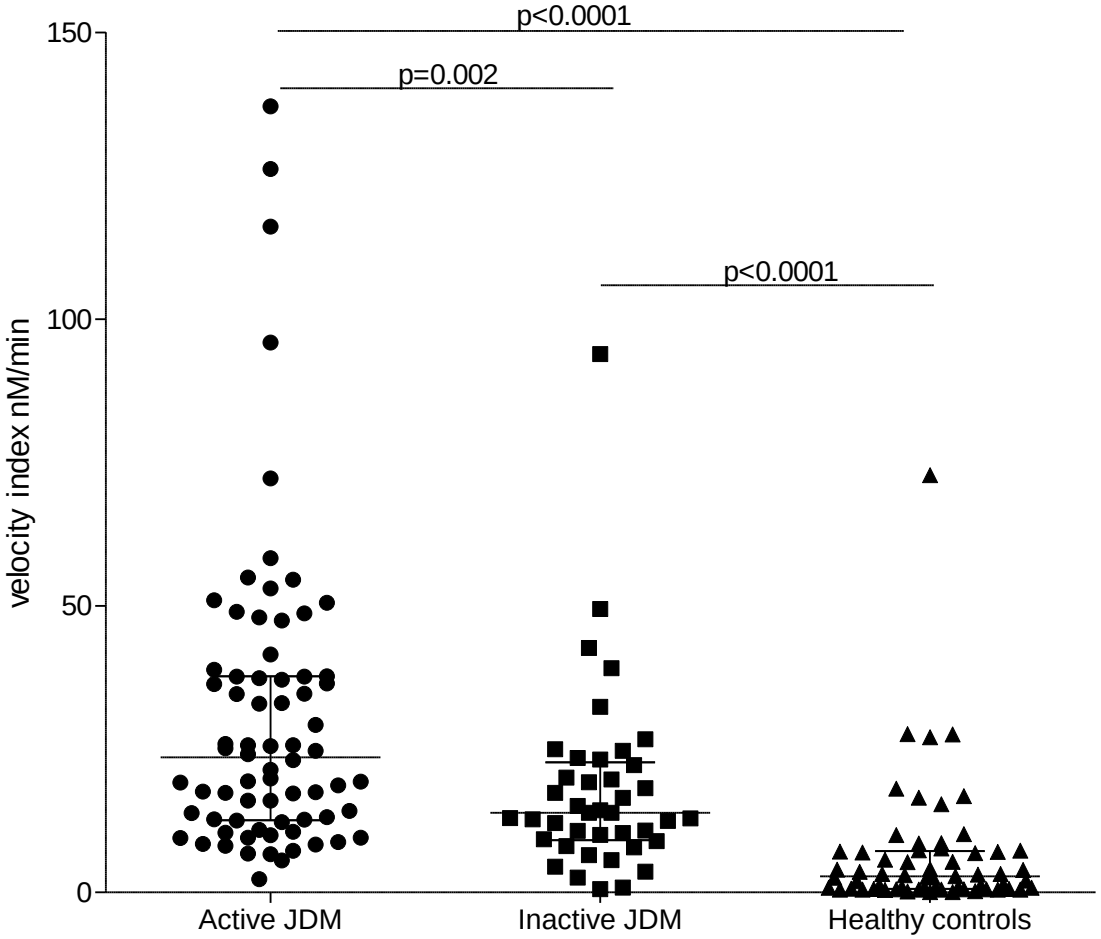

Figure 6.4. Plasma thrombin generation assay velocity index (nM/min) in children with Juvenile dermatomyositis (JDM). *Children with active JDM had increased velocity index compared with children with inactive disease, p=0.002 and healthy controls, p<0.0001. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

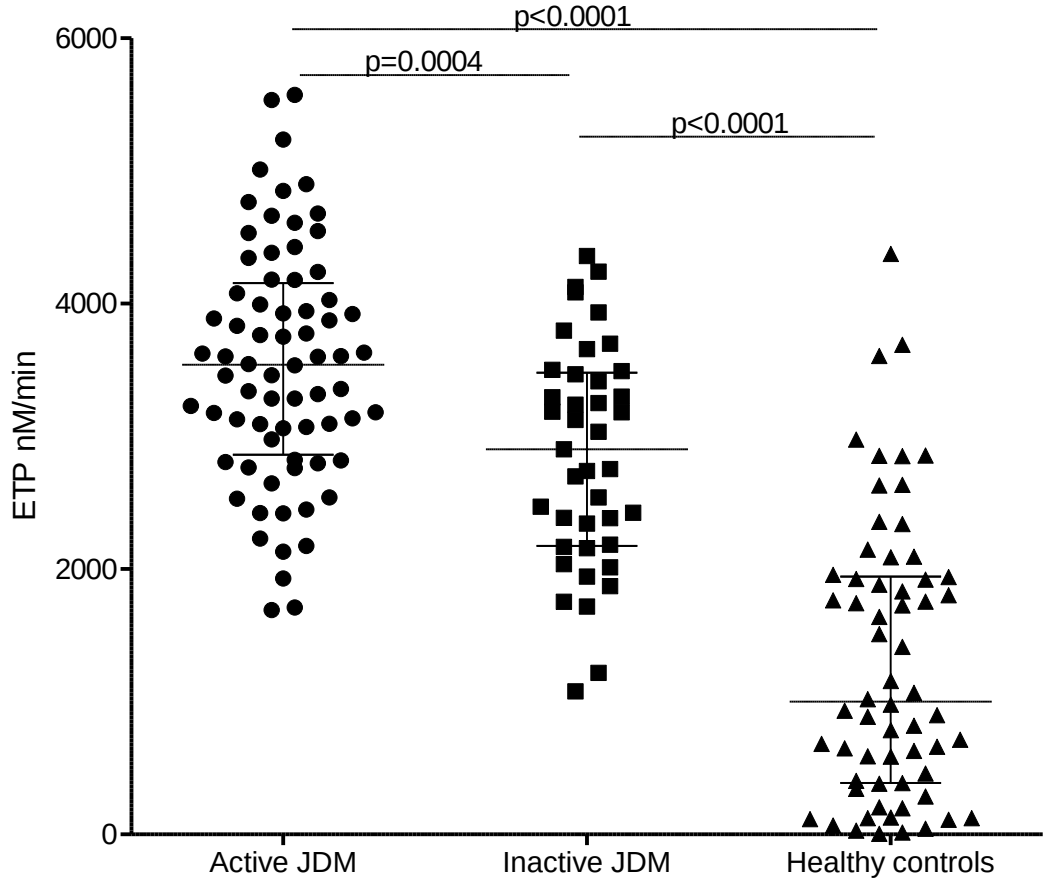

Figure 6.5. Plasma thrombin generation assay endogenous thrombin potential (ETP) nM x min in children with Juvenile dermatomyositis (JDM). *Children with active JDM had increased ETP compared to children with inactive disease, p=0.0004 and healthy controls, p<0.0001. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

6.5.2 Association of plasma TGA parameters with MP

As discussed in section 5.2, MP have strong prothrombotic properties and could thus contribute to the hypercoagulable stable observed in children with JDM. Levels of total AnV+

MP and TF+ monocyte (CD14+) derived MP (TF+CD14+AnV+ MP) were assessed in regards to the ETP and it was demonstrated that the ETP correlated significantly with the total number of plasma AnV+ MP: r=0.23, p=0.02 (**Figure 6.6**) and with the TF+CD14+AnV+ MP: r=0.29, p=0.007 (**Figure 6.7**).

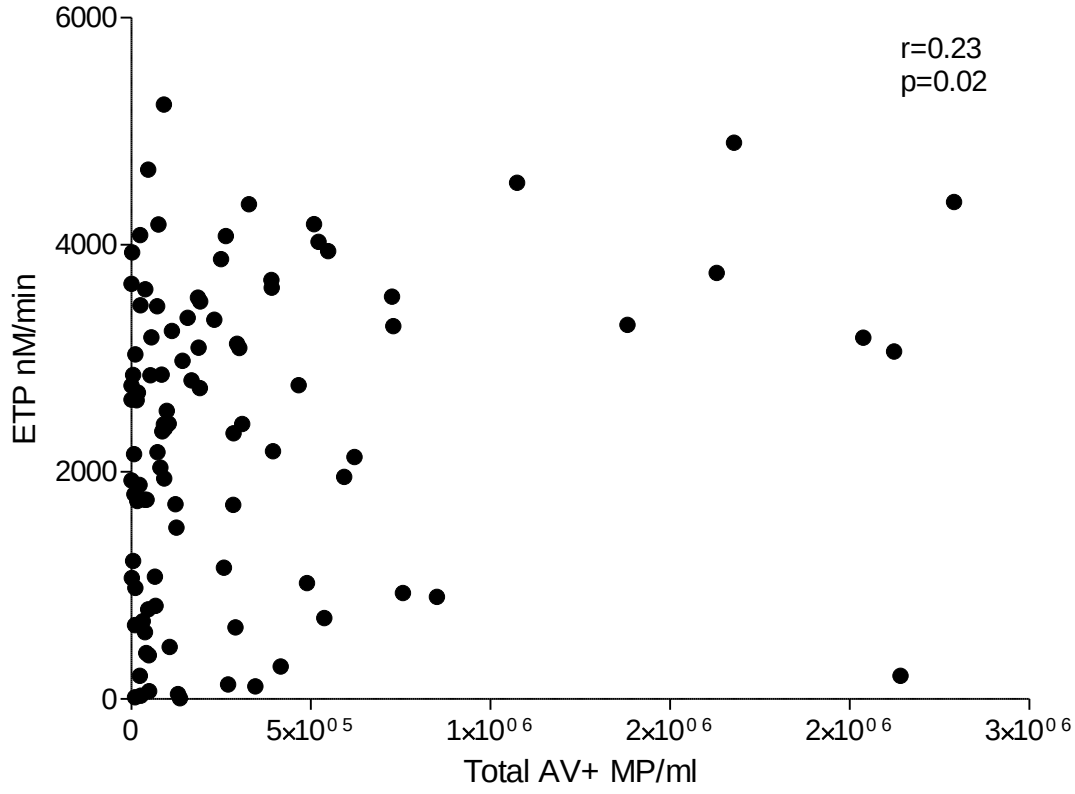

Figure 6.6. Correlation of endogenous thrombin potential (ETP) with total Annexin V+ microparticles (AnV+ MP). *ETP strongly correlated with the levels of total AnV+ MP, r=0.23, p=0.02. Correlation calculated with Spearman's correlation coefficient.*

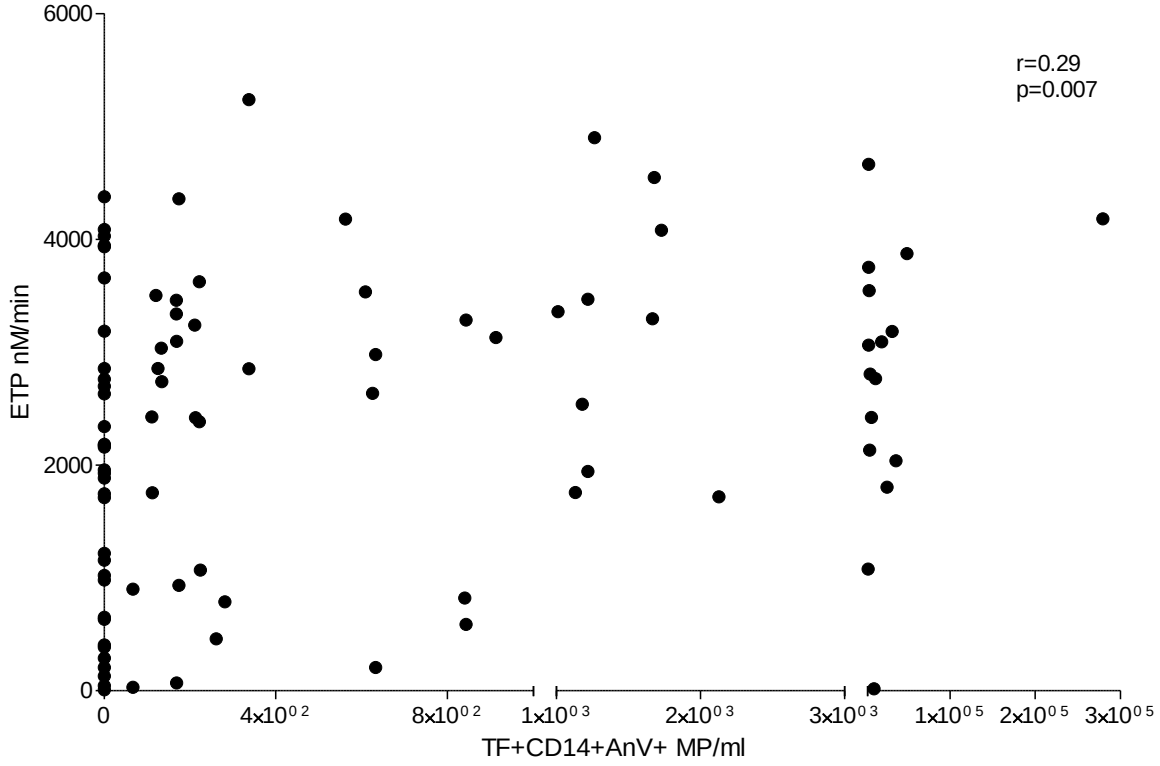

Figure 6.7. Correlation of endogenous thrombin potential (ETP) with tissue factor (TF)+ monocyte derived (CD14+) Annexin V+ microparticles (TF+CD14+AnV+ MP). *ETP strongly correlated with TF+CD14+AnV+ MP, r=0.29, p=0.007. Correlation calculated with Spearman's correlation coefficient.*

6.5.3 Association of TGA parameters with CEC

As discussed in chapter 3, CEC can detect and monitor endothelial injury. Endothelial injury with the exposure of TF can subsequently trigger the initiation of the coagulation cascade. Therefore, the correlation of CEC and ETP was examined and it was demonstrated that CEC strongly correlated with the ETP, r=0.54, p<0.0001; **Figure 6.8.**

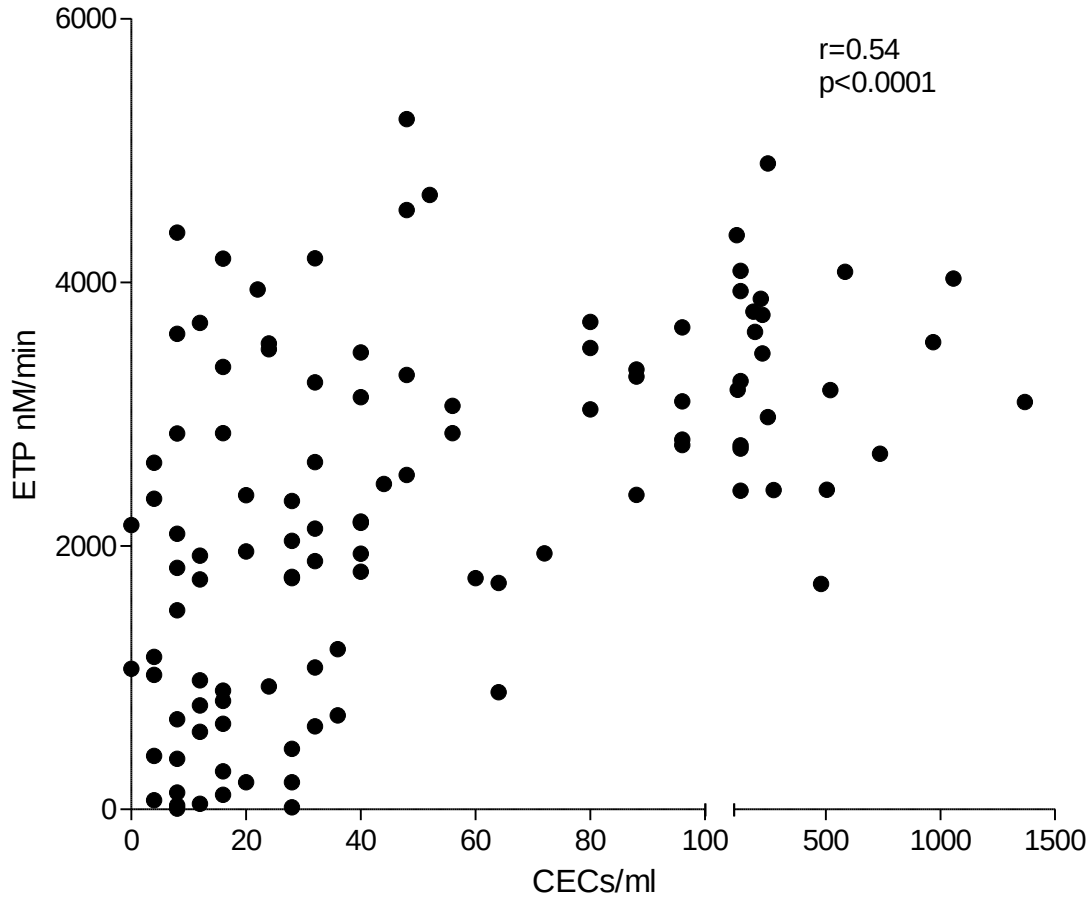

Figure 6.8. Correlation of endogenous thrombin potential with levels of circulating endothelial cells (CEC). *ETP strongly correlated with CEC, r=0.54, p<0.0001. Correlation calculated with Spearman's correlation coefficient test.*

6.5.4 Plasma peak thrombin levels in newly diagnosed JDM patients with ulcerative skin disease

As already discuss in chapter 1, approximately 23-30% of JDM cases are complicated by ulcerative skin disease. Skin ulceration is thought to mirror the end result of significant vasculopathy, caused by microinfarcts, hypoxia and ischaemia of the skin (Mamyrova *et al.*,

2007). The exact mechanism of microthrombi formulation in the skin of JDM patients has not been yet elucidated. In this chapter, plasma thrombin levels were demonstrated to be elevated in children with JDM. Thus, JDM patients were characterised by enhanced prothrombotic propensity that could potentially contribute to the development of skin ulceration. I thus thought to look into the subgroup of newly diagnosed patients and to examine plasma levels of thrombin in relation to the presence or not of ulcerative skin disease. From the 13 patients included, 12 patients were recruited at the time of diagnosis and had received no treatment. One patient with ulcerative skin disease was recruited 0.12 months post diagnosis and he had already started treatment with oral prednisolone 2mg/kg/day. Newly diagnosed patients with skin ulceration tended to have higher levels of plasma peak thrombin median 251.8 (IQR, 143.3-331.8) nM compared to newly diagnosed patients without skin ulceration with a median peak thrombin of 127.4 (IQR, 107.6-196.9) nM, but the difference did not reach statistical significance (p=0.089) probably due to small numbers; **Figure 6.9**.

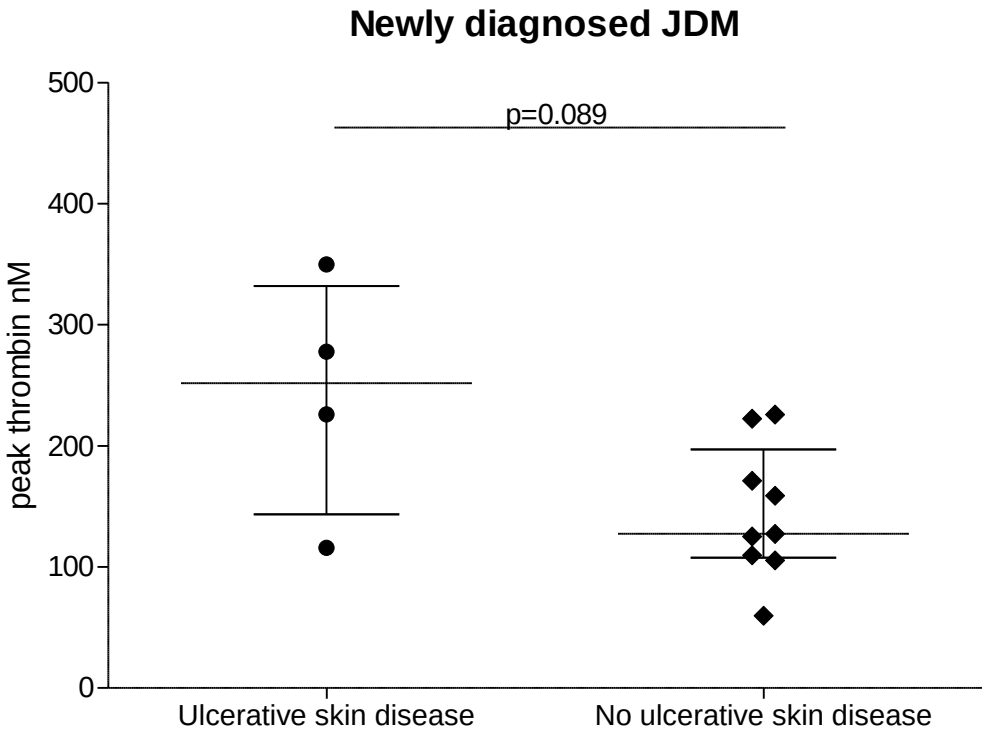

Figure 6.9. Plasma peak thrombin levels in newly diagnosed Juvenile dermatomyositis (JDM) patients based on the presence of ulcerative skin disease. *Newly diagnosed patients with ulcerative skin disease tended to have increased plasma peak thrombin compared to newly diagnosed patients without ulcerative skin disease, p=0.089. P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

6.5.5 Longitudinal changes of endogenous thrombin potential in children with JDM

Figure 6.10 summarizes the longitudinal changes of the 25 children with JDM that were studied at time 0 (time of recruitment) and during at least one follow-up visit after recruitment. Children with active JDM at time of recruitment that went into remission during the follow-up appointment (n=4 as data for one patient were missing at time of recruitment)

had no change in their ETP between the two time points from median 3234 (IQR, 3117-4232) nM/min at recruitment to median 2904 (IQR, 2647-3650) nM/min at last follow-up, p=0.41 (**Figure 6.10A**). Similarly, children with inactive disease at time of recruitment that were found to have active disease at last follow-up visit (n=2 as data for one patient were missing) had numerically lower (but not significant) ETP median 1809 (IQR, 1078-2539) nM/min at time of recruitment compared to median 3681 (IQR, 3124-4239) nM/min at last follow-up visit (**Figure 6.10B**). In the first case, the difference between the two timepoints didn't reach statistical significance while in the second case p-value could not be calculated due to the small numbers. Thus, results were inconclusive.

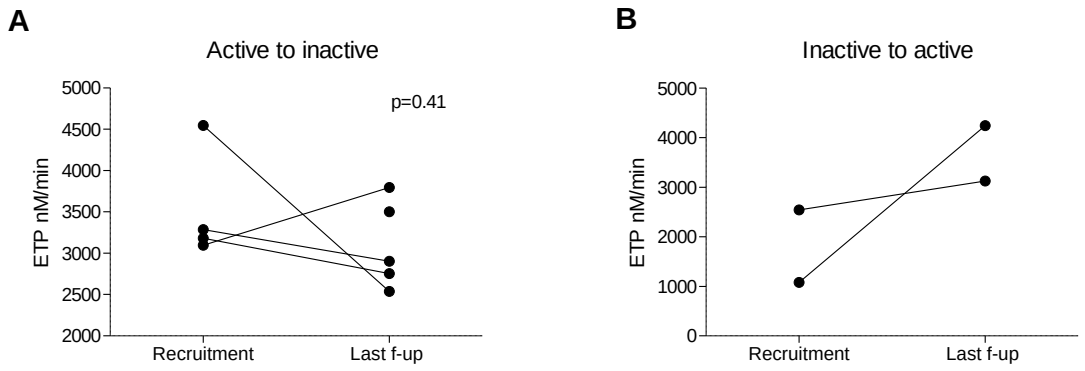

Figure 6.10. Longitudinal changes in endogenous thrombin potential (ETP) in children with Juvenile dermatomyositis (JDM). *Children with active JDM at time of recruitment that became inactive during the follow-up appointment (n=4) had no change in their median ETP between the two time points, p=0.41 (**Figure 6.10A**). Children with inactive disease at time of recruitment that were found to have active disease at last follow-up visit (n=2) had numerically higher median ETP in the latest follow-up visit (p value unable to be calculated due to low*

*numbers) (**Figure 6.10B**). Where applicable, p value calculated with Wilcoxon matched pairs signed rank test.*

6.5.6 Peak thrombin generation differentiates children with active and inactive JDM

ROC curve analysis of peak thrombin as a diagnostic test for children with active JDM was significant with an AUC of 0.72, SE 0.05 with 95% CI of 0.63-0.82, p<0.0001 (**Figure 6.11**). A cut-off peak thrombin >194.2 nM with specificity of 90.2% (95% CI of 76.9-97.2%) and sensitivity of 45.8% (95% CI of 34-58%) resulted in a positive likelihood ratio of 4.70 and negative likelihood ratio of 0.60 for children with active JDM. **Appendix 6** summarises the diagnostic test characteristics of peak thrombin generation for children with active JDM.

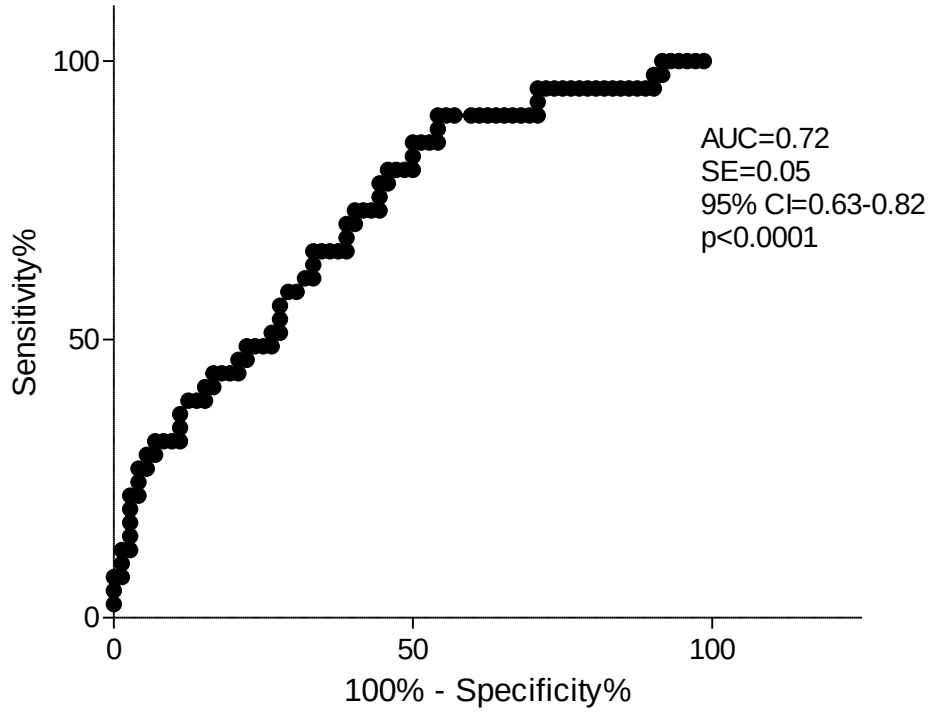

Figure 6.11. Receiver operator characteristic curve (ROC) for peak thrombin for the identification of children with active Juvenile dermatomyositis (JDM). *ROC analysis was significant with an area under the curve (AUC) of 0.72 (95% Confidence Interval 0.63-0.82), p<0.0001.*

6.6 Discussion

Children with active JDM demonstrated enhanced plasma thrombin generation when compared to children with inactive disease and healthy controls. Other parameters of the TGA including endogenous thrombin potential and velocity index were also increased in children with active disease compared to healthy controls suggesting for the first time that JDM patients are characterised by a hypercoagulable state. This prothrombotic state seems to change when the disease is well controlled, but coagulation balance is never fully restored to healthy control levels, as suggested by the observed enhanced thrombin generation also in children with inactive disease.

Vascular and perivascular inflammation is a predominant feature of JDM. Intravascular fibrin thrombi and capillary necrosis are typical histopathological features in muscle biopsies (Dalakas, 2002). Vascular ectasia and fibrin deposition are also common skin histopathological findings (Crowson and Magro, 1996). Cutaneous and visceral infarcts, whilst infrequent, do occur and are sometimes life-threatening complications in children with JDM (Papadopoulou and McCann, 2018). Although several immunological processes have been implicated in the pathogenesis of these vasculopathic features as discussed in section 1.8, the exact mechanism

contributing to micro-thrombosis in children with JDM is not fully understood. The intravascular and extravascular fibrin deposition probably results from activation of the coagulation cascade during inflammation (Smith *et al.*, 2015). Endothelial injury and damage further result in the activation of the tissue factor mediated coagulation pathway, the end product of which is thrombin. In keeping with that, in my study plasma levels of peak thrombin strongly correlated with plasma levels of circulating endothelial cells, which as discussed in chapter 3 could serve as a biomarker of endothelial injury. Apart from an important player in the coagulation cascade, thrombin also has pronounced proinflammatory effects (Esmon, 2014). Acting via the protease activated receptors (PARs) expressed in the endothelial cells, thrombin has the potential to exert leukocyte migration, cellular proliferation, regulation of vascular permeability and tone, platelet activation, and oedema formation contributing to a proatherogenic effect (Sambrano *et al.*, 2001; Reiter *et al.*, 2003; Chen and Dorling, 2009).

In my study, plasma levels of thrombin generation strongly correlated with plasma levels of total AnV+MP and TF+ monocyte derived MP suggesting for the first time another possible mechanism of hypercoagulability in children with JDM. The correlation of total AnV+MP with peak thrombin suggests that their procoagulant activity is secondary to the phosphatidylserine expressed in their surface, while the correlation of TF+ monocyte derive MP with peak thrombin indicates that monocyte derived MP can further contribute to a prothrombotic state as they offer a pool of TF in circulation. Thus, different cell derived MP contribute to a prothrombotic status in children with JDM through different mechanisms.

These results are in keeping with previous studies in which MP mediated thrombin generation was found to be altered in systemic vasculitis (Eleftheriou *et al.*, 2011), coronary artery disease (Koganti *et al.*, 2017) and in childhood stroke (Eleftheriou *et al.*, 2012).

Several studies have suggested an increased risk of thromboembolic (Zoller *et al.*, 2012a; Chung *et al.*, 2014; Carruthers *et al.*, 2016) and cardiovascular events in adults with dermatomyositis (Zoller *et al.*, 2012b; Lai *et al.*, 2013; Ungprasert *et al.*, 2014). Although, thromboembolic events are rare in children with JDM (Huemer *et al.*, 1995), there is limited evidence if children with JDM are at increased risk of thromboembolic events later in adulthood. Eimer *et al*. found increased cardiovascular risk in 8 adults with a history of JDM when compared to healthy controls (Eimer *et al.*, 2011). Of interest, in my study children with clinically inactive disease still had significantly increased plasma peak thrombin when compared to healthy controls suggesting that children with JDM continue to be on a hypercoagulable state even if the disease is deemed to be in clinical remission. This finding, in combination with the finding of chapter 3 that children with inactive disease had evidence of ongoing endothelial injury, raises the possibility that patients with JDM could be at increased risk of cardiovascular and thrombotic events later in adulthood. Hypercoagulable state and ongoing endothelial injury may have a cumulative effect. There is a big gap in this field, and prospective studies are required: it may be that such patients may benefit from prophylactic antithrombotic therapy, although this remains entirely speculative at this stage.

The findings of my study can have important clinical and therapeutic implications not only by better understanding disease pathogenesis but also by suggesting possible new therapeutic targets. Micro- and macrovascular complications in JDM resulting from an ongoing prothrombotic state suggests that anti-thrombin agents may have a place in the treatment of JDM, especially for patients with severe vasculopathic features. Blockade of thrombin activity may not only restore the coagulation balance but also have an anti-inflammatory effect contributing to better disease control. In this regard, a study in mice demonstrated that the oral thrombin inhibitor, dabigatran, had marked anti-inflammatory and anti-fibrotic effects in a bleomycin model of pulmonary fibrosis suggesting a new therapeutic approach for the treatment of interstitial lung disease (Bogatkevich *et al.*, 2011). Moreover, hirudin, another thrombin inhibitor, has been demonstrated to reduce synovial inflammation in antigen induced arthritis (Varisco *et al.*, 2000). Thus, a selective group of JDM patients that demonstrate high plasma levels of circulating MP and peak thrombin may benefit from a thrombin inhibitor, rather than more aggressive immunosuppression. Prospective clinical trials with long follow up would be required to answer that question, however, and would be extremely challenging to power adequately since cardiovascular events are rare.

My study has several limitations. The effect of treatment towards a prothrombotic state has not been assessed. About one third of the studied population has been on corticosteroids, and may bias the findings because, although controversial, corticosteroids might contribute to a prothrombotic state (van Zaane *et al.*, 2010). A meta-analysis demonstrated that their effect differs based on the clinical condition for which they are used (van Zaane *et al.*, 2010).

Moreover, included patients were on a different combination of therapeutic agents and had different disease and treatment duration and thus subgroup analysis was not possible due to small numbers. To overcome that limitation, newly diagnosed and treatment naive patients were included in a subgroup analysis which demonstrated that children with skin ulceration tended to have enhanced thrombin generation, but again results did not reach statistical significance due to small numbers. On the other hand, studying children has the advantage that they do not have any of the traditional thrombotic risk factors such as smoking, alcohol or drug-abuse. The effect of the MP on thrombin generation was speculated based on their observed correlation, and the biological plausibility of this mechanism based on previous work (Eleftheriou *et al.*, 2011). The direct effect of MP on the coagulation cascade was not directly assessed in my study, however, an area that is worthy therefore of future study.

Regardless of these study limitations, my study suggests that children with active JDM have enhanced thrombin generation characteristic of a hypercoagulable state. MP may be important determinants. Although the prothrombotic state improves with treatment, it never fully normalises suggesting a possible increased risk of cardiovascular and thrombotic events later in adulthood. Confirming these findings in multinational prospective studies is crucial to better understand the interactions between coagulation and the immune system in JDM shedding light into disease mechanisms and allowing the development of innovative therapeutic interventions.

7 Arterial stiffness in children with Juvenile dermatomyositis

7.1 Summary

Background: JDM is a systemic inflammatory disease that presents in childhood with progressive muscle weakness and pathognomonic skin rashes. Vasculitis and occlusive vasculopathy are central to the pathogenesis, causing significant morbidity and mortality. Virtually nothing is known about the burden of cardiovascular disease (CVD) in JDM.

Objectives: To examine if the JDM vasculopathy in concert with traditional cardiovascular risk factors, contributes to increased arterial stiffness in paediatric patients with JDM as a surrogate of an increased risk of cardiovascular disease.

Methods: Sixty-five patients recruited to the UK JDM Cohort & Biomarker Study were included; median age 11.4 (IQR, 7.4 – 14.0) years with median disease duration of 3.0 (IQR, 1.3-7.0) years. Forty-one (63.1%) were females. JDM patients were compared with 72 healthy controls; median age 15.9 (IQR, 10.7-17.6) years; 40 (55.6%) were females. Carotid-femoral and carotid-radial pulse wave velocity (PWV) were assessed with oscillometry using the Vicorder device (Skidmore Medical Limited). Analysis of covariance was used to compare the slope of PWV versus age between the groups using linear regression.

Results: Carotid-radial PWV in relation to age slope in JDM patients (0.44m/sec/year, Y intercept 3.414) differed significantly from the healthy control slope (0.12m/sec/year, Y intercept 5.903, p=0.003), suggesting increased arterial stiffness in JDM patients. There was no difference when carotid-femoral PWV in relation to age was compared between children with JDM and healthy controls.

Conclusion: Increased arterial stiffness was detected in children with JDM, suggestive of premature atherosclerosis, although the prognostic significance of this remains uncertain.

7.2 Introduction

Systemic inflammatory diseases are some of the most prevalent chronic health conditions in the UK constituting a major cause of morbidity, physical impairment and increased use of the health care services (Cooper *et al.*, 2009; van Halm *et al.*, 2009). The coexistence of traditional cardiovascular risk factors, long-term use of corticosteroids, endothelial dysfunction and vascular injury, and chronic inflammation creates the perfect environment and chain reaction for early atherogenesis in this population (van Halm *et al.*, 2009). For instance, adults with severe RA have increased risk for cardiovascular events similar to the risk seen in diabetes mellitus (van Halm *et al.*, 2009; Lindhardsen *et al.*, 2011). A meta-analysis showed that patients with RA had 50% higher risk of cardiovascular disease and mortality compared to the general population (Avina-Zubieta *et al.*, 2012). Mortality rate is three times higher in SLE compared to the general population which is mainly attributed to premature atherosclerosis (Recio-Mayoral *et al.*, 2009). Two meta-analyses showed that patients with systemic sclerosis have increased carotid-intima media thickness, a marker of early atherosclerosis, compared to healthy controls (Tyrrell *et al.*, 2010; Au *et al.*, 2011). Regarding idiopathic inflammatory myopathies, in a large general population study, Rai *et al*. observed a nearly 4- and 3-fold increased risk of myocardial infarction in patients with PM and DM, respectively, compared to those without, even when adjusted for known risk factors (medications, gender, age and utilisation of outpatient healthcare services) (Rai *et al.*, 2016). A systematic review and meta-

analysis published in 2014 demonstrated an increased coronary artery disease in patients with IIM with a pooled risk ratio of 2.24 (95% CI: 1.02–4.92) (Ungprasert *et al.*, 2014).

Traditional cardiovascular risk factors, including obesity, hyperlipidemia, hypertension, smoking and diabetes, are more prevalent in patients with autoimmune diseases (Bruce, 2005; Chung *et al.*, 2012; Diederichsen *et al.*, 2015), but do not account for the whole effect (Bruce, 2005). Long-term use of corticosteroids, a key therapy in most autoimmune diseases, is associated with worsened hyperglycaemia, hyperlipidaemia and obesity, contributing to accelerated atherosclerosis. The role of corticosteroid treatment in atherosclerosis in autoimmune disease is debatable, however, with some studies suggesting that better control of inflammation through corticosteroid treatment reduces progression of atherosclerosis (Svenungsson *et al.*, 2001; Roman *et al.*, 2003; Bruce, 2005). Other immunosuppressive treatments have also been implicated in the pathophysiology of atherosclerosis including azathioprine and ciclosporin (Miller, 2002). Chronic inflammation has been well described to be related to atherogenesis and possibly atherosclerosis with IL-1β having a dual role both as a proinflammatory but also as an atherogenic cytokine (Matsuura *et al.*, 2014). Finally, endothelial dysfunction is a central link between risk factors and initiation, progression and complications of the atherogenic process (Sitia *et al.*, 2010).

Several inflammatory conditions begin in childhood. An estimated of 1 in 10,000 children in the UK are affected by an inflammatory arthritis each year (Cooper *et al.*, 2009). On this basis, the frequency of systemic inflammatory diseases in childhood is likely to be much higher. Due

to therapeutic advances made in the last decade, the majority of these children will now survive into adulthood and are likely to face many novel emerging health concerns including increased morbidity and mortality from cardiovascular disease. Virtually nothing is known about the burden of CVD in JDM. In chapters 3, 4 and 5 children with JDM were found to have ongoing endothelial injury as demonstrated by increased plasma levels of CEC, endothelial related cytokines, adhesion molecules and EMP. Moreover, in chapter 6, children with JDM were found to have a hypercoagulable state which improved with treatment and disease control but never returned to healthy control levels. These observations, in combination with studies suggesting that JDM patients have altered cardiovascular risk factors (hyperlipidemia, increased insulin resistance, obesity, hypertension) (Coyle *et al.*, 2009), suggest that children with JDM may be at increased risk of early atherosclerosis later in adulthood. This is further supported by the only study conducted in adults with a history of JDM, demonstrating increased carotid-intima media thickness when compared to healthy controls (Eimer *et al.*, 2011). Early detection of accelerated atherosclerosis, and risk of late cardiovascular morbidity in this paediatric patient population is thus crucial in order to allow clinicians to introduce preventative strategies as early as possible to reduce the risk of cardiovascular events in early adult life. Understanding the mechanisms underpinning the accelerated atherosclerosis may also help in identifying novel, targeted, preventative therapies.

7.2.1 Measures of endothelial dysfunction and arterial stiffness as surrogate markers of accelerated atherosclerosis.

The annual incidence of cardiovascular or cerebrovascular events in children with rheumatic disease is very low, and therefore too small to adequately power studies that can be completed in a reasonable time-frame. Additionally, detecting preclinical atherosclerosis has been highlighted as a priority in order to instrument primary preventive strategies, which are currently not part of routine JDM clinical care. A good way to achieve this is by using surrogate markers of atherosclerosis. Studies in adults have established that early cardiovascular disease can be detected using a range of methodologies including carotid-intima-media thickness (CIMT), pulse wave velocity (PWV), and measures of endothelial vasodilator function such as the ENDO-PAT stress test (Vincze *et al.*, 2014). These are described in more detail below.

1. CIMT measures the thickness of media and intima layers of the carotid artery (the two inner layers). A recent systematic review and meta-analysis has shown that CIMT can be used as a predictor of future vascular events in otherwise healthy adults. In particular, it was shown a 10-15% and 13-18% increased risk of future myocardial infarction and ischaemic events (strokes), respectively, in the presence of an absolute CIMT difference of 0.1 mm (Lorenz *et al.*, 2007).

2. The most commonly used modality for the assessment of arterial stiffness is PWV, a non-invasive and reproducible way of measuring early changes in arterial wall stiffness. A recent systematic review demonstrated that aortic stiffness as assessed with PWV is a strong

predictor of future cardiovascular events: subjects with increased aortic PWV had two times higher risk for cardiovascular events and related mortality (Vlachopoulos *et al.*, 2010).

3. The Endo-PAT 2000 is a relatively new device that is used to assess endothelial vasodilator function in a rapid and non-invasive fashion. The device assesses endothelial-mediated reactive hyperaemia by quantifying changes in the blood volume in the finger (via peripheral arterial plethysmography) in response to a 5-minute occlusion of the arteries in the upper limb. Recently it has been demonstrated that assessment of endothelial function by non-invasive peripheral arterial tonometry using Endo-PAT stress tests also accurately predict late cardiovascular adverse events (Rubinshtein *et al.*, 2010).

In summary, a number of indices can be employed to non-invasively assess endothelial responses and peripheral arterial stiffness as surrogate markers of atherosclerosis.

7.3 Aim

To investigate whether children with JDM have increased arterial stiffness and other structural arterial changes indicative of early atherosclerosis.

7.4 Methods

7.4.1 Measurement of arterial stiffness

To assess structural arterial disease, carotid-femoral and carotid-radial pulse wave velocity (PWV) was used as a marker for arterial stiffness. PWV was assessed with oscillometry using

the Vicorder device (Skidmore Medical Limited). During each heart beat a pulse wave travels from the heart down the arterial wall in advance of blood flow. The subject reclines on a bed with their back at 30° from horizontal and is asked not to speak and remain still to avoid increases in the arterial tone caused by the influence of speaking or moving. A cuff is put gently around the neck, the wrist and the upper part of the femur to sensor the carotid, the radial and femoral artery respectively (**Figure 7.1**). The distance between the three cuffs is measured by drawing a straight line that connects the three studied sites (cm). Vicorder is used to measure the delay (sec) of the pulse waveform between the carotid artery and femoral and radial artery, caused by arterial distension. The more rigid the arteries are, the less the capability of distention they demonstrate and thus the faster the waveform is travelling within the arteries resulting in a smaller time delay and increased pulse wave velocity. Increased PWV correlates with more extensive atherosclerotic changes to the arteries and worse arterial health (Tillin *et al.*, 2007). The time delay (ms) in combination with the straight-line distance between the cuffs (cm) provides PWV in ms-1. All measurements were taken in duplicates and the average value was used as per the American Heart Association recommendations (Urbina *et al.*, 2009).

Figure 7.1. Demonstration of the Vicorder device*. Three cuffs are placed around the neck to sensor the common carotid, the thigh to sensor the femoral artery and the wrist to sensor the radial artery. Image adapted from SMT medical GmbH&Co website (https://www.smt-medical.com).*

7.4.2 Study population

Children with JDM and healthy controls were recruited at GOSH and UCLH as previously described in section 2.1.1 and 2.1.2, respectively. A separate information leaflet was given with information regarding cardiovascular studies and possible complications. Consent form was obtained from parents/guardians or participants where appropriate. For both groups, age, height and weight were recorded before vascular studies were performed. Blood

pressure and heart rate were also measured at the brachial artery by sphygmomanometry with an appropriate size-cuff and the value was used in the analysis. Clinical and laboratory indicators of disease activity were assessed concurrently at the time of the study.

7.4.3 Statistical analyses

As it has been previously demonstrated that PWV correlates positively and significantly with age (Cheung *et al.*, 2002), analysis of covariance was used to compare the slope of PWV versus age between the groups using linear regression. Multiple linear regression analysis was performed to identify possible predictors of carotid-radial PWV. Statistical analyses were performed using Stata (release 7.0) and GraphPad Prism, version 5.03 (San Diego, CA).

7.5 Results

Sixty-five patients recruited to the UK JDM Cohort & Biomarker Study were included in this study; median age 11.4 (IQR, 7.4 – 14.0) years with median disease duration of 3.0 (IQR, 1.3-7.0) years. Forty-one (63.1%) were females. JDM patients were compared with 72 healthy controls; median age 15.9 (IQR, 10.7-17.6) years; females were 40 (55.6%). Traditional cardiovascular risk factors for JDM patients and healthy controls are presented in **Table 7.1**. It is important to highlight at that point, none of the JDM studied patients and the healthy controls were smoking or drinking alcohol. As it has been previously reported that PWV correlates with age (Cheung *et al.*, 2002; Shah *et al.*, 2015), I firstly investigated if that observation was also true for my study population. In keeping with previous reports, both

carotid-radial and carotid-femoral PWV strongly and positively correlated with age when all study groups were included (r=0.48, p<0.0001 and r=0.71, p<0.0001) (**Figure 7.2**).

Risk factors	**JDM patients**	**Healthy controls**	**p-value**
Body Mass Index	19.5 (15.7-22.7)	20.5 (17.0-23.2)	0.15
Systolic blood pressure in relation to age slope (mmHg/year), r^2	0.15	0.10	0.99
Triglycerides, mmol/L	0.96 (0.67-1.20)	0.77 (0.58-1.06)	0.15
Cholesterol, mmol/L	3.7 (3.4-4.2)	4.4 (3.7-4.8)	**0.003**
All values are expressed as median with interquartile range.			

Table 7.1. Traditional cardiovascular risk factors in children with Juvenile dermatomyositis (JDM) compared to healthy controls. *P values were calculated using Mann Whitney U test. Results are presented as median and interquartile range.*

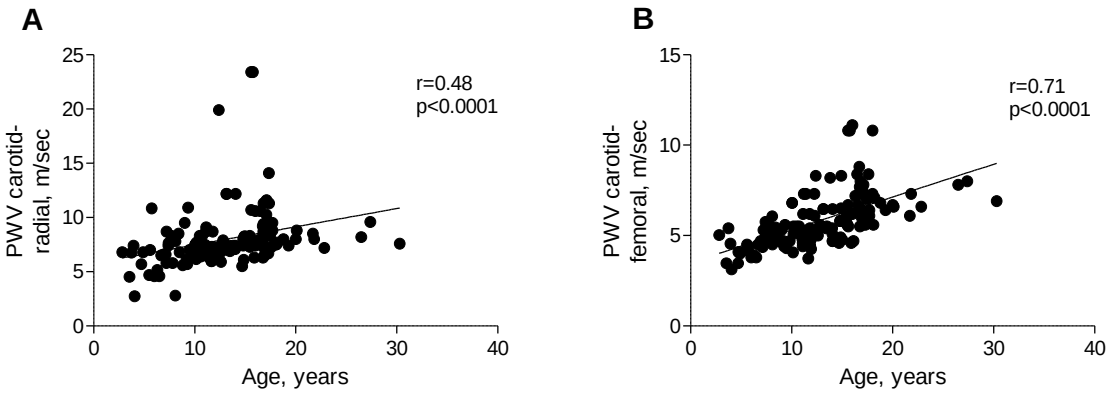

Figure 7.2. Correlation of pulse wave velocity (PWV) with age in all subjects included in this study (Juvenile dermatomyositis patients and healthy controls). A. *Carotid-radial PWV correlated strongly with age (r=0.48, p<0.0001).* ***B.*** *Strong correlation demonstrated between carotid -femoral PWV and age (r=0.71, p<0.0001). Correlations were calculated with Spearman's correlation coefficient test.*

Carotid-radial PWV in relation to age slope in JDM patients (0.44m/sec/year, Y intercept 3.414) differed significantly from the healthy control slope (0.12m/sec/year, Y intercept 5.903, p=0.003), suggesting increased arterial stiffness in JDM patients (**Figure 7.3**). When carotid-femoral PWV in relation to age slope was measured, JDM patients' slope (0.25m/sec/year, Y intercept 2.971) did not differ compared to healthy controls' slope (0.17m/sec/year, Y intercept 3.483), p=0.12 (**Figure 7.4**).

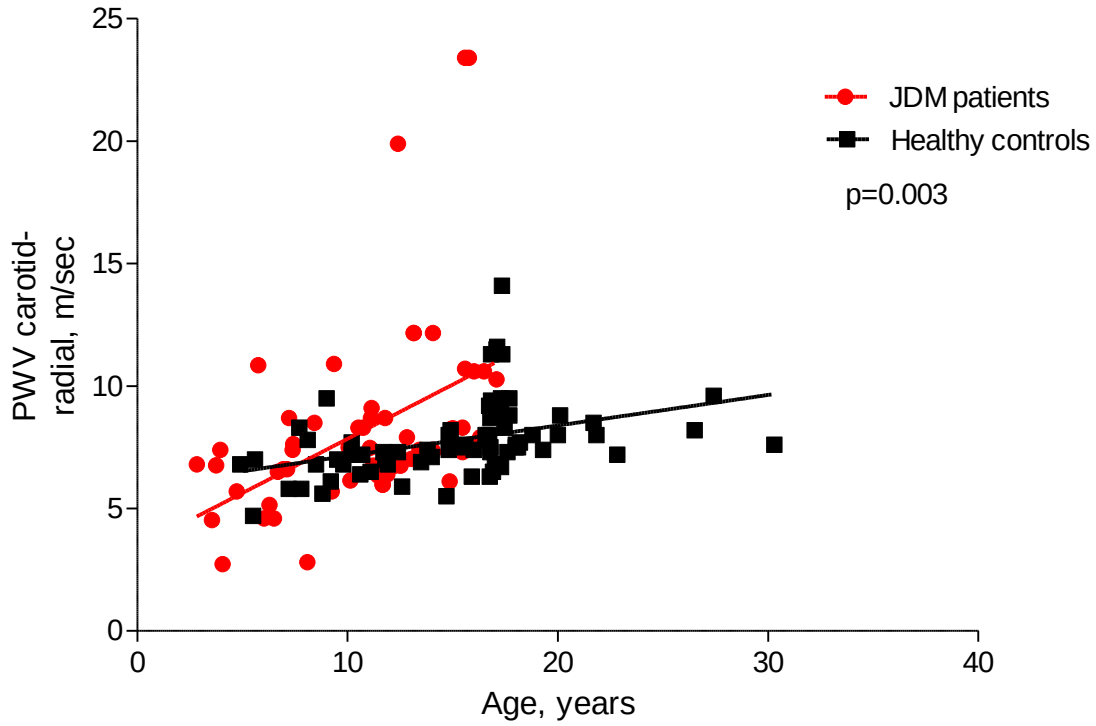

Figure 7.3. Carotid-radial pulse wave velocity (PWV) in relation to age slope in Juvenile dermatomyositis (JDM) patients compared to healthy controls. *A significant difference between the two slopes was observed, p=0.003. P-value was calculated with linear regression.*

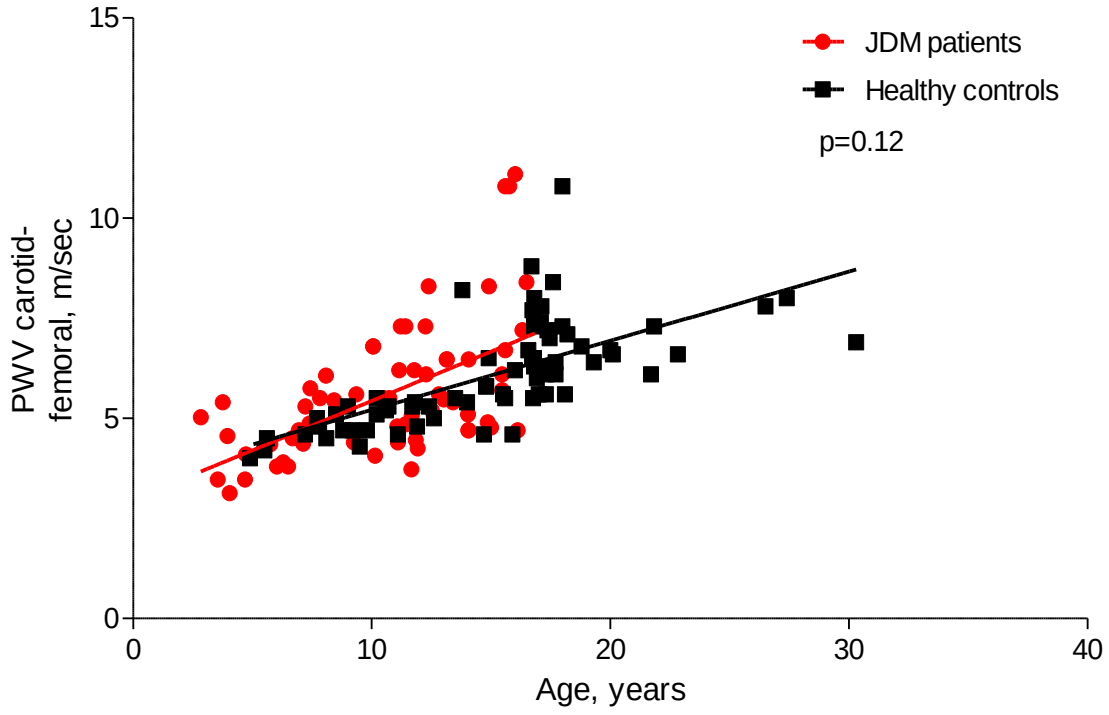

Figure 7.4. Carotid-femoral pulse wave velocity (PWV) in relation to age slope in Juvenile dermatomyositis (JDM) patients compared to healthy controls. *No significant difference was identified between the two groups, p=0.12. P value was calculated with linear regression.*

I then decided to examine which of the study parameters affect PWV in a multiple regression analysis. Age, sex, disease duration at time of recruitment, abnormal nailfold capillaries, disease activity, CEC, ETP, galectin-9, total AnV+MP, TNFRII were included in the multiple regression analysis. From the variables included, only age significantly correlated with the carotid-radial PWV values, p=0.0022 (**Table 7.2**).

Independent Variables	Coefficient	Standard Error	T	P	$r_{partial}$	$r_{semipartial}$
(Constant)	3.3329					
Age	0.6385	0.1886	3.385	**0.0022**	0.5458	0.5194
Sex	-2.7171	1.3697	-1.984	0.0575	-0.3567	0.3044

Table 7.2. Multiple linear regression analysis predicting carotid-radial pulse wave velocity (PWV) in children with Juvenile dermatomyositis (JDM). *From the variables included in the regression model, age was the only one that strongly correlated with the carotid-radial pulse wave velocity values, p=0.0022.*

7.5.1 Longitudinal changes of carotid-radial pulse wave velocity in relation to age slope in children with JDM

Of the 25 JDM children that were studied prospectively, 15 children had repeated carotid-radial PWV at last follow-up visit. As demonstrated in Figure 7.5, carotid-radial PWV in relation to age slope tended to increase at the last follow-up visit (slope 0.34m/sec/year, Y intercept 4.999) compared with the carotid-radial PWV in relation to age slope at the time of recruitment (slope 0.27, Y intercept 4.657) but the difference did not reach statistical significance (p=0.88) probably due to the small numbers. Similarly, in regard to the carotid-femoral PWV in relation to age slope, there was no difference between the time of recruitment (slope 0.20, Y intercept 3.307) and the last follow-up visit (slope 0.30, Y intercept 2.515), p=0.49 (**Figure 7.6**).

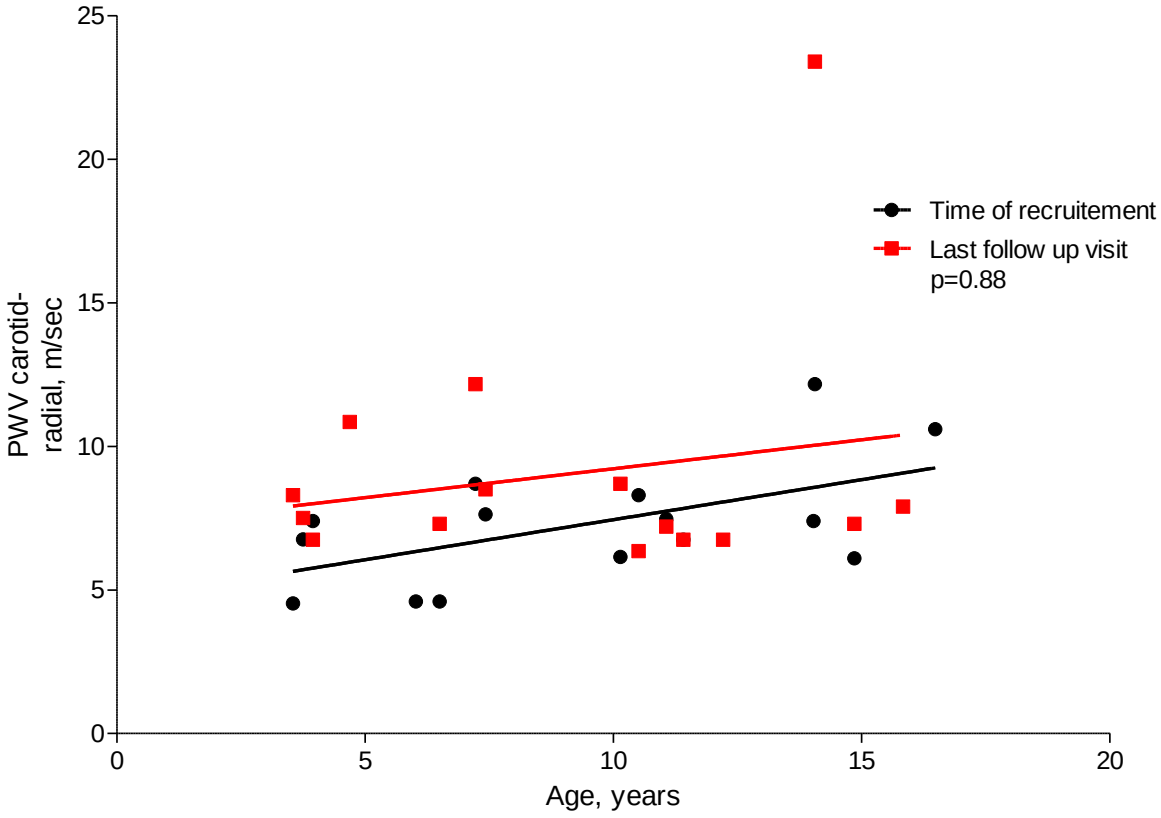

Figure 7.5 Longitudinal changes of carotid-radial pulse wave velocity (PWV) in relation to age slope in children with Juvenile dermatomyositis (JDM). *There was no significant difference between the carotid-radial PWV in relation to age slope at the time of recruitment compared to the last follow-up visit, p=0.88. P value calculated with linear regression.*

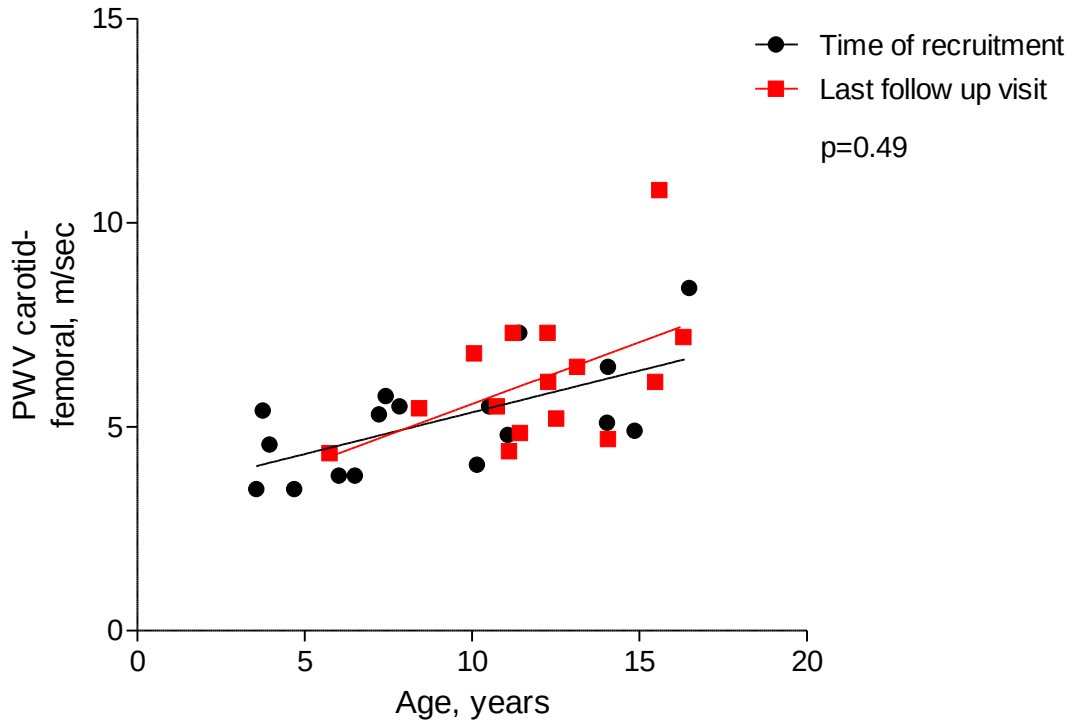

Figure 7.6. Longitudinal changes of carotid-femoral pulse wave velocity (PWV) in relation to age slope in children with Juvenile dermatomyositis (JDM). *There was no significant difference between the carotid-radial PWV in relation to age slope at the time of recruitment compared to the last follow-up visit, p=0.49. P value calculated with linear regression.*

7.6 Discussion

Children with JDM were found to have increased carotid-radial PWV in relation to age slope when compared to healthy controls suggestive of increased arterial stiffness in this study group. That effect persisted in the small group of patients studied prospectively suggesting (for the first time) that children with JDM may develop structural arterial changes during the disease course that could indicate early onset atherosclerosis and increased risk of

cardiovascular events later in adulthood. When traditional risk factors were assessed, there was no difference between the two studied groups with the exception of cholesterol (JDM patients, perhaps counterintuitively, had decreased cholesterol levels compared to healthy controls), further suggesting that the observed increase in the arterial stiffening represents the cumulative effect of the disease not only in small but also in larger arteries.

PWV is one of the most frequently used modalities used as a surrogate non-invasive marker of atherosclerosis. The European Society of Cardiology suggested in 2006 PWV as "the simplest, most robust, non-invasive and reproducible method for the assessment of the artery stiffening" (Laurent *et al.*, 2006). In adults, PWV is strongly related with the development and the extent of atherosclerosis and probably consists an independent predictor of cardiovascular events (Blacher *et al.*, 1999). In contrast with adults, few data are available in children (Cheung *et al.*, 2002; Cheung *et al.*, 2004). Moreover, although PWV has been suggested by the American Heart Association as the gold standard method for the assessment of arterial stiffness in children (Urbina *et al.*, 2009), validation and reproducibility studies have not yet been conducted in the paediatric and adolescent population (Urbina *et al.*, 2009). The main obstacle for the conduction of such studies is that findings would need to be confirmed with invasive techniques. Although, PWV has not been validated in paediatric populations, there is no good explanation why this technique should be less effective or reproducible in paediatric patients compared to the adult population.

Carotid-femoral PWV which mainly reflects structural arterial changes in aorta was not found to be increased in children with JDM when compared to healthy controls. One could speculate that children with JDM might have increased carotid-femoral PWV as the results of ongoing endothelial injury, chronic inflammation, hypercoagulable status and long-term use of corticosteroids. The absence of such an observation could be due to the short time of median disease duration of 1.63 (IQR, 0.28-4.66) years. That could theoretically explain why although children with JDM were not found to have increased carotid-femoral PWV, adults with a history of JDM have been previously demonstrated to have enhanced FMD when compared to healthy controls (Eimer *et al.*, 2011). On the other hand, an interesting and unexpected finding of this study was that carotid-radial (but not carotid-femoral) PWV in relation to age slope was found to be increased in the JDM patients. Carotid-radial PWV mainly assesses the peripheral arterial stiffness of upper limb muscular arteries (branchial and radial arteries), while carotid-femoral PWV is a marker of central arterial (aortic, i.e. elastic artery) stiffness. It is well described that different arterial segments respond differently to aging and other factors that contribute to arterial stiffness, probably related to differences in elastin-collagen smooth muscle proportions, with more studies suggesting a bigger effect in the more proximal, more elastic, and less muscular arteries (van der Heijden-Spek *et al.*, 2000). As it has been previously prescribed JDM is mainly a small vessel vasculitis/vasculopathy characterised by proliferation of the intima of small vessels, thrombosis and infarction (Rosa Neto and Goldenstein-Schainberg, 2010). The underlying pathophysiology of the disease may, thus, explain this unexpected finding. Another possible explanation is that increases in carotid-radial PWV seen in patients with JDM may reflect the effect of proximal muscle

inflammation in the peripheral arteries. Previous studies suggest that both carotid femoral and carotid radial PWV may predict coronary artery events (Rohani *et al.*, 2005; Young-Soo *et al.*, 2006). Finally, several studies have suggested that carotid-radial PWV reflects microvascular endothelial dysfunction (McCall *et al.*, 2010; Badhwar *et al.*, 2018). Thus, changes in carotid-radial PWV may be associated not with structural arterial changes but with functional increase in smooth muscle tone resulting in an increased peripheral arterial stiffness. Further studies need to be carried out trying to shed light on the clinical significance of this finding. It would be also of great interest to investigate whether this is a sustained effect prospectively.

When possible confounding factors were assessed, carotid-radial PWV was found to correlate with age but not with other known cardiovascular risk factors or with previously studied indices of endothelial dysfunction including abnormal nailfold capillaries, CEC, ETP, galectin-9, total AnV+MP and TNFII. Although PWV is known to be associated with age and blood pressure, varying correlation coefficients have been reported in studies (Amar *et al.*, 2001; Weber *et al.*, 2004). Nailfold capillaroscopy is a surrogate marker of the small vessel vasculitis that characterises JDM. Moreover, as previously demonstrated, nailfold capillary changes relate with ongoing skin disease and cardiac complications in children with JDM (Christen-Zaech *et al.*, 2008). The fact that carotid-radial PWV was not found to correlate with nailfold capillaries abnormalities could be explained by different mechanisms affecting PWV and nailfold capillaries. As previously discussed, carotid-radial PWV possibly reflect functional changes in medium size arteries while nailfold capillaries changes are structural changes of

small size arteries. Moreover, the lack of association between PWV and laboratory markers of endothelial dysfunction could be explained by the fact that CEC, ETP, galectin-9 and TNFRII reflect the state of the endothelium at the time of the sample collection in contrast with PWV that possibly mirror the cumulative effect over time to medium size arteries of several factors including disease per se, treatment and chronic inflammation. Further multiple linear regression models investigating more risk factors (genetic factors, MSAs, high density lipoprotein and low density lipoprotein) should be developed trying to identify the best predictive model for increased arterial stiffness in children with JDM.

Recently, several studies have focused their interest into the role of MP not only as markers but also as significant contributors to atherosclerosis. MP of different cellular origins have been demonstrated to be associated with the Framingham risk score, a score which estimates the risk of heart disease in 10 years (Ueba *et al.*, 2008; Nozaki *et al.*, 2009). Moreover, there is a strong correlation between MP and traditional cardiovascular risk factors including smoking (Heiss *et al.*, 2008), hypertension (Preston *et al.*, 2003), obesity (Goichot *et al.*, 2006). Additionally, MP are known to play a significant role in inflammation (Mesri and Altieri, 1999; Tesse *et al.*, 2005; Scanu *et al.*, 2008), endothelial function (Brodsky *et al.*, 2004; Agouni *et al.*, 2008), angiogenesis (Kim *et al.*, 2004; Leroyer *et al.*, 2008) and thrombosis (Sabatier *et al.*, 2002; Scholz *et al.*, 2002) which subsequently are known promoters of atherogenesis. MP have also been shown to represent transport vehicles of miRNAs and other active proteins, such as bone morphogenic proteins, with a possible important role in regulating the formulation of atherosclerotic plaque (Buendia *et al.*, 2015) and evolution toward plaque

instability and rupture (Cipollone *et al.*, 2011; Raitoharju *et al.*, 2011). In chapter 5, children with JDM were found to have increased levels of circulating MP further supporting that children with JDM may be at risk of early atherosclerosis later in adulthood.

My study has several limitations. First of all, not all patients included in the cross-sectional study had PWV, that may have contributed to a selection bias. That was mainly because of technical difficulties as younger children, usually below the age of 5.5 years old, were sometimes afraid of having the cuff placed around their neck and were becoming unsettled when the cuffs were inflated, which made the measurement of PWV impossible. Thus, only older patients were included with a longer disease duration. Moreover, cholesterol levels measured in JDM patients represent fasted levels in contrast with cholesterol levels in healthy controls which were unfasted levels. That could potentially explain the paradoxal finding of JDM children having lower cholesterol levels when compared to healthy control. The effect of different therapeutic regimes on PWV was not assessed mainly because of the numerous combinations of different therapeutic regimes of included patients which made a subgroup analysis impossible.

In summary, this study demonstrated that patients with JDM had increased upper limb peripheral arterial stiffness compared to healthy controls; the significance of this remains uncertain, although this could have significant prognostic implications for the future. Further studies are needed to try to investigate the clinical significance of this observation.

8 Janus kinase 1/2 inhibition with baricitinib in the treatment of Juvenile Dermatomyositis

8.1 Summary

Objectives: Although the overall prognosis of JDM has significantly improved over the recent years, the long-term outcome differs substantially between patients despite current therapeutic strategies. No drug currently targets both the systemic inflammatory component and the vasculopathic component of the disease, processes that play a central role into the disease pathogenesis. Baricitinib is a JAK 1/2 inhibitor that blocks IFN signalling. As IFNs drive the endothelial injury of JDM, JAK inhibition may play a role in therapy of JDM vasculopathy.

Methods: This is a report of refractory JDM case treated with baricitinib as part of the open-label compassionate use treatment program. Different validated tools and indices were employed to capture the extent of disease activity pre-treatment and one, six, twelve- and eighteen-months post baricitinib treatment. CEC, STAT signalling and IFN gene expression were also evaluated.

Results: Treatment with baricitinib led to significant improvement in measures of disease activity, including modified skin DAS, parent and physician Global assessment. There was a concomitant reduction in the serum levels of IFN responses. Baricitinib also significantly reduced the levels of CEC, although these did not return back to normal values.

Conclusions: Baricitinib (JAK 1/2 blockade) might be an effective treatment in refractory JDM skin disease, through modulation of immune responses and improvement of vasculopathy.

8.2 Introduction

The overall prognosis of JDM has improved significantly over recent years, but the long-term outcome differs substantially from patient to patient (Harris-Love *et al.*, 2009; Ravelli *et al.*, 2010), perhaps suggestive of distinct clinical prognostic phenotypes. Because of its complexity and clinical heterogeneity, treatment of JDM remains challenging. Therapeutic approaches of JDM mainly consist of non-specific anti-inflammatory or immunosuppressive medications. Corticosteroids are the first line treatment, with methotrexate being the DMARD of choice used for over 30 years (Fisler *et al.*, 2002; Guseinova *et al.*, 2011; Ruperto *et al.*, 2016). Ciclosporin (Ruperto *et al.*, 2016), azathioprine (Jacobs, 1977), mycophenolate mofetil (Kawasumi *et al.*, 2015; Fasano *et al.*, 2016), intravenous immunoglobulin (Mimori *et al.*, 2007; Kim *et al.*, 2017b) and cyclophosphamide (Riley *et al.*, 2004; Kawasumi *et al.*, 2015) are employed for resistant to treatment disease, or those with major organ involvement. Recently, rituximab (Oddis *et al.*, 2013) and anti-TNF agents (Campanilho-Marques *et al.*, 2016b) have entered clinical practise in JDM. However, this approach to treatment is associated with significant side effects, and may be ineffective in some patients. Considering these limitations, alternative therapies are needed.

It is now increasingly recognised that JDM falls into the category of diseases driven by interferons collectively referred to as “interferonopathies” (Bilgic *et al.*, 2009; Reed *et al.*, 2012). One of the characteristic histopathological findings in patients with DM, the presence

of tubuloreticular inclusions in muscle endothelial cells (Norton *et al.*, 1970; Banker, 1975), has been recognised as the end effect of type I IFN signalling (Grimley *et al.*, 1985; Kuyama *et al.*, 1986). Moreover, transcriptomic studies on muscle biopsy specimens from DM patients have demonstrated significant upregulation of type I IFN stimulated genes (Salajegheh *et al.*, 2010; Suarez-Calvet *et al.*, 2014) which has also been confirmed at protein level (Greenberg *et al.*, 2005; Suarez-Calvet *et al.*, 2017). Similar findings have been also confirmed in DM skin biopsies (Wenzel *et al.*, 2005; Wong *et al.*, 2012). Additionally, type I IFNs (Liao *et al.*, 2011; Rodero *et al.*, 2017) and type I IFN related cytokines have been shown to be elevated in the peripheral blood both in children and adults with DM (Greenberg, 2010; Bellutti Enders *et al.*, 2014), while IFN stimulated gene expression in the peripheral blood of DM patients has also been suggested to strongly relate with disease activity (Greenberg *et al.*, 2012). There is thus strong evidence to suggest that the IFN pathway plays a crucial role in JDM pathogenesis, and blockade of this pathway may represent a novel therapeutic target in this group of patients.

All 5 types of type I IFNs bind to IFN-α receptor, a heterodimeric receptor, and signal is then transduced to the nucleus through the JAK/STAT pathway resulting in the upregulation of numerous IFN stimulated genes (Ivashkiv and Donlin, 2014). JAKs, including JAK1, JAK2, JAK3 and tyrosine kinase (TYK)2, are intracellular tyrosine kinases that upon activation have the ability to either phosphorylate themselves, or phosphorylate other JAKs (O'Shea *et al.*, 2013). Upon activation by cytokines, STATs bind to phosphorylated receptors resulting in their phosphorylation through JAKs. The STATs then dimerize and translocate to the nucleus where

they initiate relative gene transcription (Schwartz *et al.*, 2016). There are seven STATs: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6; each one of them interacts with specific JAKs and transduces signalling of specific cytokines (Schwartz *et al.*, 2016). JAK inhibitors have proven to be an effective therapeutic approach in many autoimmune and autoinflammatory diseases where upregulation of the interferon signaling is thought to play a key role in disease pathogenesis (Clark *et al.*, 2014). Moreover, JAK inhibitors have been successfully used in other interferonopathies, notably the proteasome associated autoinflammatory syndromes that clinically and immunologically share similar features with JDM (Liu *et al.*, 2012). Furthermore, there is now increasing evidence that JAK inhibitors may be effective in adults with DM as demonstrated by a recently published case series (Kurtzman *et al.*, 2016; Ladislau *et al.*, 2018; Moghadam-Kia *et al.*, 2019). Hitherto, there is only one report of tofacitinib, a JAK 1/3 inhibitor, used in a child with severe JDM with good response (Aeschlimann *et al.*, 2018). Baricitinib is an oral small molecule that inhibits JAK 1/2 activation, thereby reducing downstream circulating cytokine levels and activation of dendritic cells and T-cells (O'Shea *et al.*, 2013; Xing *et al.*, 2014) that are known to play an important role in JDM pathogenesis.

In this chapter, I describe a paediatric case of refractory JDM, not responsive to biologic therapies, treated with baricitinib as part of the open-label compassionate use treatment program (14V-MC-JAGA) that provided baricitinib to patients with conditions expected to benefit from JAK inhibition, resulting in significant improvement in skin disease and blood biomarker changes (Papadopoulou *et al.*, 2019).

8.3 Methods

An 11.5-year-old boy with resistant to treatment JDM was included in an open-label compassionate use treatment program (14V-MC-JAGA, expanded access protocol NCT01724580). Baricitinib was administered according to a standard protocol. Informed consent was obtained (ethics approval 08H071382, 11/LO/0330 and MREC/1/3/22).

8.3.1 Assessment of disease activity

Different validated tools and indices were employed to capture the extent of disease activity: CMAS, MMT-8, PhyGLOVAS, functional ability through the CHAQ, modified skin DAS and Par GLOVAS. The following laboratory markers of disease activity was measured in each study visit: ESR, CRP, CK, ALT, AST and LDH.

8.3.2 Assessment of endothelial injury

CEC were isolated and enumerated with immunomagnetic bead extraction as previously described in section 2.2.4.

8.3.3 IFN stimulated gene RNA expression in total blood

Blood for IFN stimulated gene assessment was collected into PAXgene tubes (PreAnalytix, Hombrechtikon, Switzerland) and, after being kept at room temperature for between 2 and

72 h, was frozen at −20°C until extraction. Total RNA was extracted from whole blood with a PAXgene (PreAnalytix) RNA isolation kit as per manufacturer instructions. RNA concentration was assessed with a spectrophotometer (FLUOstar Omega, Labtech, Ortenberg, Germany). Quantitative reverse transcription polymerase chain reaction (qPCR) analysis was performed using the iTaq Universal SYBR Green Supermix (172-5121, Bio-Rad), and cDNA derived from 400 ng total RNA. Using Qiagen Quantitec primers for IFI27 (HS_IFI27_1_SG), IFI44L (HS_IFI44L_1_SG), IFIT1 (HS_IFIT1_1_SG), ISG15 (HS_ISG15_1_SG), RSAD2 (HS_RSAD2_1_SG), SIGLEC1 (HS_ISG15_1_SG), CXCL10 (HS_CXCL10_1_SG), signal transducer and activator of transcription factor 1 (STAT1) (Sigma Forward primer: TGCTTGGATCAGCTGCAGAA; Reverse primer: CCGAACTTGCTGCAGACTCT), the relative abundance of each target transcript was normalised to the expression level of *HPRT1* (Hs03929096_g1) and assessed using the CFX Maestro software.

8.3.4 STAT-1 and STAT-3 phosphorylation

8.3.4.1 PBMC isolation

PBMC were isolated from blood collected into Falcon tubes (Thermo Fisher Scientific) containing 40 U preservative free heparin (CP Pharmaceuticals) per 50 ml and processed within 2 hours of collection. Blood was then diluted with an equal volume of RPMI 1640 medium and overlaid on an equal volume of Lymphoprep TM (Axis Shield), and then centrifuged at 800 g for 10 min with the brake off. The PBMC layer was taken, resuspended in an equal volume of culture medium and centrifuged at 500g for 10 minutes. The pellet was then resuspended in warm RPMI with 10% Fetal Calf Serum (FCS). The suspension was again

centrifuged at 500g for 10 minutes and the resulting pellet was resuspended in appropriate volume of freezing medium at a concentration of 2 x10^6 /ml, aliquoted in 1 ml and transferred into individual cryovials which were placed into a freezing pot with isopropanol coolant for 24 hours at -80^{o}C (allowing slow cooling) over night before transfer to liquid nitrogen storage for future use.

8.3.4.2 Quantification of viable cells

For the quantification of viable cells, 10 µl of 0.4% trypan blue (Sigma) were added to 10µl of cell suspension. Half of the resulted mix was then placed on Neubauer counting chamber and cells enumerated by light microscopy. The total count of unstained live cells was calculated in the specified 25-box field. The total number of live cells per ml was then calculated with the following equation: number of cells in 25-box field x 2 (dilution factor) x 10^4.

8.3.4.3 Cell stimulation and staining

Cryo-preserved cells were thawed in a 37^{0}C water bath for 2 minutes. Warm RPMI 1640 medium was then added drop-by-drop into the cryovial containing the cell suspension, slowly over a 40-second period. The diluted cell suspension was then transferred to a 50-mL polypropylene centrifuge tube containing 8 mL of warm RPMI 1640 medium for every vial of cells added. Cells were then centrifuged (200g for 7 minutes) before being counted. Cell pellets were then resuspended in 1 ml of RPMI 1640 medium and counted using the Trypan blue method and diluted to a final working concentration of 4-5 x10^6 PBMC/mL. Two hundred µl of the final diluted sample were then plated into a 96 well plate and incubated for 3 hours

to allow for cell packing recovery. Each cell suspension was either not stimulated or stimulated with IFN-α (5ng/mL) for 30 minutes and activation was stopped by the addition of Lyse/Fix Buffer (BD Biosciences, San Diego, CA) following the manufacturer's protocol. Samples were run in duplicates. Cells were then centrifuged at 200g for 3 minutes and cell pellets were re-suspended in staining buffer. Cells were stained with the cell surface markers BV711-CD4 (Biolegend, cat: 317440, OKT4, 1:100), BV605-CD14 (Biolegend, cat: 301833, M5E2, 1:100) and BV510-CD8a (Biolegend, cat: 301048, RPA-T8, 1:100) and PE-Cy7-CD19 (Biolegend, cat: 363012, SJ25C1, 1:100) for 1h at room temperature protected from light. Cells were then washed and centrifuged as previously described. They were then fixed using BD Cell Fix Buffer (10 min at 37^{o}C). Cells were then washed, permeabilized in ice-cold Phosflow Perm Buffer III (BD Biosciences, San Diego, CA) for 30 minutes at 4°C in the dark and then FACS buffer (PBS, pH 7.4 (ThermoFisher Scientific, Waltham, MA), 1% BSA, 4mM E DTA, 0.2% sodium azide) was added. Further staining was then performed with PE-anti-STAT1 (BD Bioscience, cat: 612564, pY701, 1:50) and Alexa Fluor® 488-anti-STAT3 (BD Bioscience, cat: 557814, 4/P-STAT3, 1:50).

8.3.4.4 Flow cytometry analysis of STAT-1 and STAT-3 phosphorylation

Flow cytometry analysis was performed on a BD LSRII flow cytometer. All samples were transferred to FACS tubes before being analysed by flow cytometry. Two additional samples were also prepared to serve as negative controls. For the negative control tube, 200 µL of FACS Buffer were added to one tube without staining with any antibodies. Samples were acquired on the low flow speed setting for 30s, obtaining roughly 1x10^{5} events per sample.

Specific gating for each antibody was achieved by using appropriate isotype controls. Gating was conducted as shown in **Figure 8.1**: First, cell subtypes were identified based on their forward scatter area (FSC-A) and side scatter area (SSC-A) properties and on surface antigen expression. Cell subtypes were defined as follows: CD14+cells as monocytes, CD3+cells as Cd3+ T-cells, CD4+ cells as Cd4+ T-cells and CD19+cells as B-cells. Same strategy was used both for stimulated and unstimulated samples. Results were analysed using FlowJo v10.4.2 with median fluorescence intensity (MFI) of the corresponding phosphorylated STATs (pSTATs) being calculated for each cell subset.

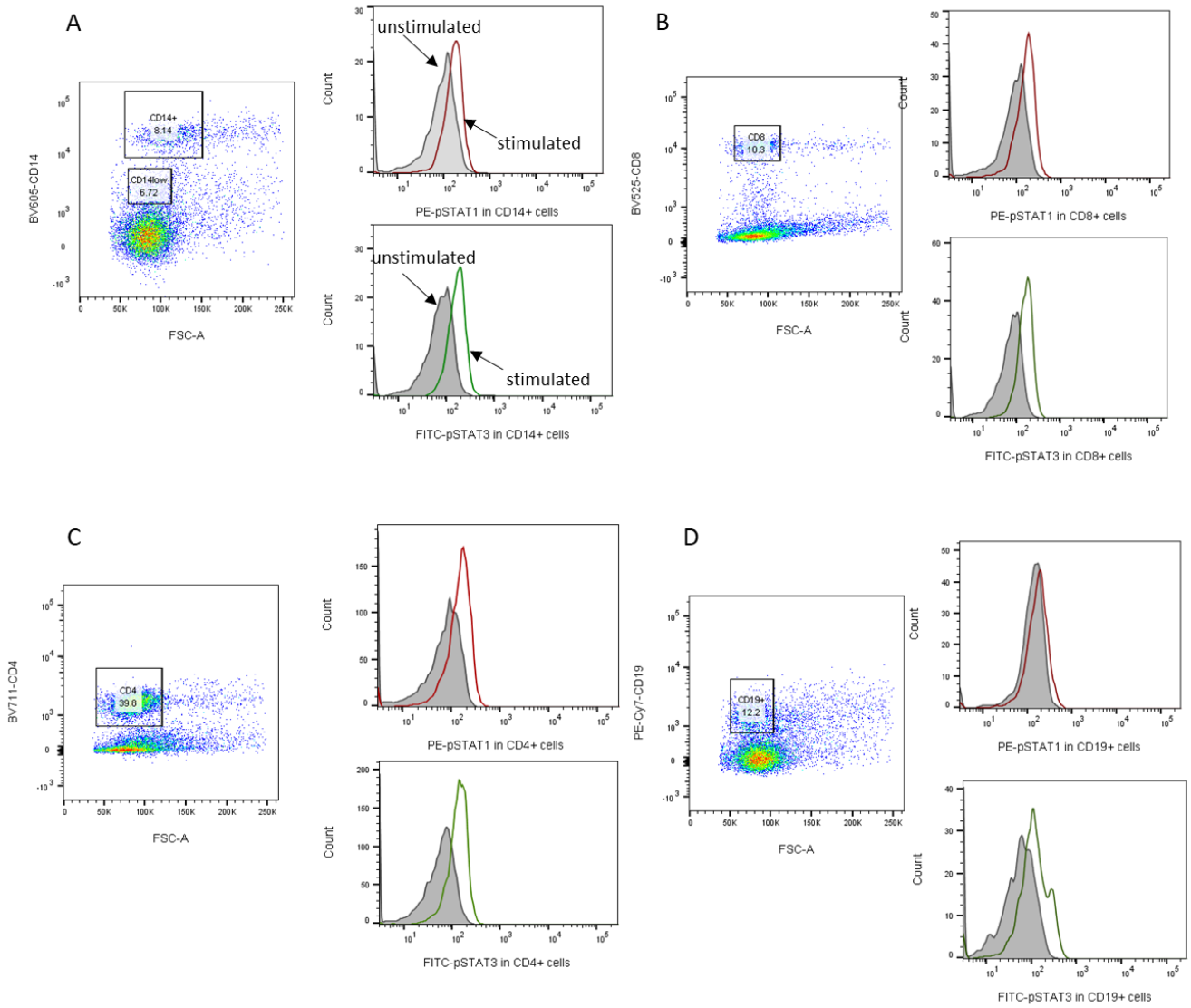

Figure 8.1 Gating strategy used in the analysis of intracellular signaling pathways in peripheral blood mononuclear cells (PBMC). *Cell subtypes were defined based on surface antigen expression as monocytes (CD14+ cells,* ***Figure 2.5A****), as CD8+ T-cells (CD3+ cells,* ***Figure 2.5B****), as CD4+ T-cells (CD4+ cells,* ***Figure 2.5C****) and as B-cells (CD19+ cells,* ***Figure 2.5D****). Each cell subtype was then examined for intra-cellular staining for p-STAT1 (PE+ cells) and p-STAT3*

(FITC+ cells); see histograms. Cells were examined at resting conditions and following stimulation with IFN-a.

8.3.4.5 Vasculitis and Inflammation Panel (VIP) targeted genetic sequencing

Genetic analysis was performed by Dr Ebun Omoyinmi using the VIP targeted next generation genetic sequencing. Detailed description of the development and validation of this panel has been previously described (Omoyinmi *et al.*, 2017). A list of genes included in the panel is provided in **Appendix 7**. In brief the capture of targeted genes/regions was performed using the Agilent QXT Target Enrichment system according to the manufacturer's protocol (Version B.2, October 2014) for Illumina sequencing. Briefly, genomic DNA was sheared by enzyme fragmentation, and ligated with SureSelect Adaptor Oligo Mix. Fragment size was assessed using the TapeStation 2100 Bioanalyzer (Agilent Technologies). The adaptor ligated libraries were then amplified and hybridized to our customized SureSelect panel. Captured libraries were indexed (barcoded), pooled and sequenced as multiplex of 16 samples on the benchtop next generation Illumina MiSeq sequencer in 150bp paired-end mode according to the standard protocol for this platform. Read alignment, variant calling, and annotation were performed using bioinformatics pipelines. Identified variants were classified according to pathogenicity, evaluated for coverage and visually inspected using the Integrative Genomics Viewer (Broad Institute).

8.4 Results

An 11.5 year-old Caucasian male with persistent refractory to treatment JDM was enrolled in the open-label compassionate use program (expanded access protocol NCT01724580). He was diagnosed with JDM at the age of 2.5 years. At presentation, he had severe muscle weakness with nasopharyngeal involvement requiring nasopharyngeal tube due to unsafe swallowing (CMAS of 18/52; MMT-8 of 56/80). He also had significant skin disease with characteristic heliotrope rash, Gottron's papules over the small and large joints (skin DAS of 4/5). He had raised CK up to 403U/L (Reference range RR 6-330U/L) and LDH up to 1509U/L (RR 450-770U/L); ESR was mildly elevated (ESR= 18 mm/h, RR < 10 mm/h) and CRP was normal (CRP=3 mg/L, RR< 20 mg/L). He had no evidence of interstitial lung disease with normal lung function tests and imaging of his chest and no evidence of cardiac involvement with normal echocardiogram and electrocardiogram. Apart from mild constipation, there was no evidence of significant gastrointestinal tract involvement. Testing for myositis specific antibodies was positive for TIF1g and Ro-52 antibodies. Further autoimmune workup demonstrated positive ANA (titer 1;640), negative double stranded DNA, normal C1q, C3 and C4 levels and normal complement function. He was initially treated with pulses with IV MEP (30mg/kg IV methylprednisolone for 3 days) followed by oral prednisolone 2-3 mg/kg/day with a plan to wean over 4-5 months and subcutaneous methotrexate (15mg/m^2 once a week). Because of poor response to initial treatment and development of significant ulcerative skin disease (modified skin DAS 5/5), early in disease course he received 6 doses iv cyclophosphamide (500-750 mg/m^2 given every 2-3 weeks to a total of 6 doses) with only partial response (CMAS 44/52 and modified skin DAS 3/5). Because of recalcitrant skin disease

and development of calcinotic lesions over his elbow joints, right ear lobe and left knee joint, he received several treatments over the next seven years, including ciclosporin, azathioprine, mycophenolate mofetil, infliximab, adalimumab, rituximab, tacrolimus and IVIG with partial or no response. Skin disease was never fully controlled and he became steroid dependent (2 mg/kg/day of prednisolone), as his skin disease flared up every time corticosteroid dose was weaned down. Due to his poor response to treatment, to exclude any monogenic interferonopathies that could mimic JDM, genetic testing was performed for the 166 targeted gene panel (VIP) (Omoyinmi *et al.*, 2017) which did not demonstrate any pathogenic class 5 or class 4 variants.

Because of recalcitrant skin disease and development of calcinosis, at the age of 11.5 years old, he was started on baricitinib (a selective JAK 1/2 inhibitor) initially at a dose of 6mg mane and 3mg nocte. No dose-related side effects were observed, and the dose was gradually escalated to 6 mg twice a day after 4 weeks according to a pre-specified treatment protocol based on weight and renal function (**Appendix 8**). At 6 months, there was an improvement in his disease activity scores compared to baseline as follows: CMAS improved from 46/52 at time of start of therapy to 50/52 at 6 months of treatment; MMT-8 improved from 59/80 to 70/80; modified skin DAS from 5/5 to 1/5; PhyGLOVAS from 4.3/10 to 1.5/10 (**Figure 8.2**). Calcinotic lesions remained stable. Patient reported outcomes also improved; CHAQ from 1.75 to 0.125; pain VAS from 6.3/10 to 2.1/10; parental VAS from 4/10 to 2/10. CK remained within normal limits ranging between 70-109 U/L. Corticosteroid dose was tapered from a

prednisone equivalent dose of 1.7 mg/kg/day before starting baricitinib to 0.3 mg/kg/day at 6 months. No significant adverse events were reported.

Despite weaning down corticosteroid dose, he sustained the improvement in his symptoms until 12 months post baricitinib initiation, when he had a flare of his disease due to poor compliance (all medication was stopped against medical advice). Six weeks after stopping his treatment, there was a significant deterioration in all disease activity parameters; modified skin DAS 5/5, CMAS 46/52, MMT-8 59/80, PhyGLOVAS 6/10 (**Figure 8.2**), CK elevated at 925 IU/L and there was worsening myalgia and arthralgia. Baricitinib was then re-started and within 2 weeks significant clinical improvement was again observed with disease activity parameters at the latest follow-up visit at 18 months post baricitinib as follows: CMAS 52/52, MMT-8 78/80, modified skin DAS 1/5, PhyGLOVAS 1/10, CK 155 IU/L (**Figure 8.3A and 8.3B**). Of note, within 18 months of treatment with baricitinib an increase in his growth was observed with his weight increasing from 60.4 kg (91st centile for age) to 77.4 kg (99th centile for age); and height from 148.6 cm (75th centile) to 154. 8 cm (91st centile for age).

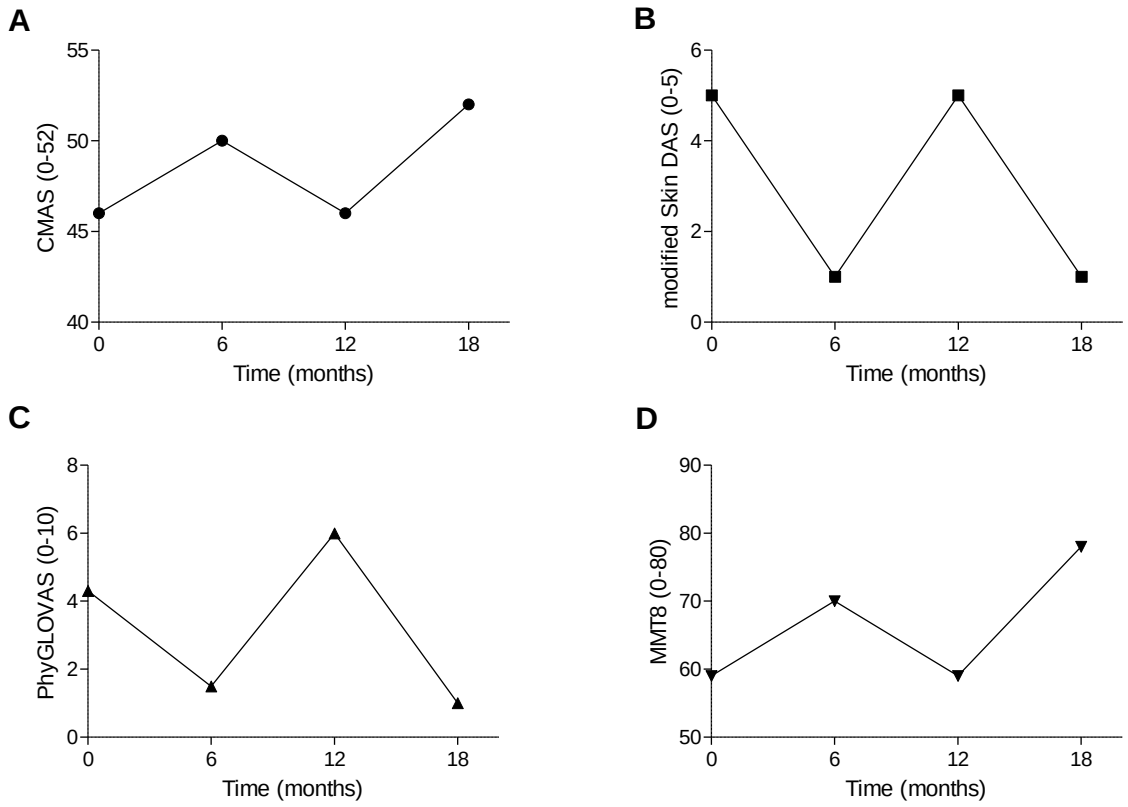

Figure 8.2 Changes in Childhood Myositis Assessment Scale (CMAS), modified skin Disease Activity Score (DAS, range 0-5), Physician's global visual Analogue Scale (PhyGLOVAS, range 0-10) and Manual Muscle Test (MMT) 8 for a patient with Juvenile dermatomyositis (JDM) treated with baricitinib at the time treatment was started (time 0), 6 months, 12 months and 18 months later.

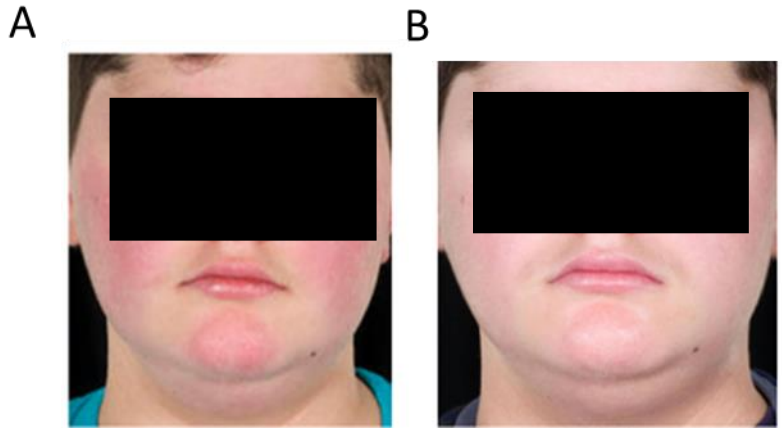

Figure 8.3. An 11.5 years old patient treated with baricitinib due to recalcitrant skin disease (A-B). *Facial cutaneous manifestations were significantly improved 18months after starting treatment with baricitinib (8.3 B) compared to baseline (8.3 A).*

To investigate how changes in clinical symptoms and disease activity parameters reflect in biomarkers of IFN signaling, the type I IFN related gene expression and STAT1 in peripheral blood were measured at the different timepoints. It is important to highlight that at the time that baricitinib treatment was started, the patient was on a high dose of corticosteroids (prednisolone equivalent of 1.7mg/kg/day) which explains the only modest upregulation of type I IFN related gene expression observed at baseline. Type I IFN related gene expression and STAT1 significantly decreased at 6 months and 18 months post baricitinib treatment compared to baseline with an increase observed in 12 months when all medication was stopped against medical advice (**Figure 8.4**). STAT1 phosphorylation was then measured to assess responsiveness of type I IFN receptor during baricitinib treatment; changes in STAT1 phosphorylation in CD4+, CD8+ and CD14+ cells were similar to those observed in type I IFN

related gene expression. STAT1 phosphorylation in all cell types decreased at 6- and 18-months post baricitinib treatment with an increase observed at 12 months when all medication was stopped due to poor compliance (**Figure 8.5 A-C**). Finally, to assess the effect of baricitinib treatment on JDM-related vasculopathy, levels of CEC were measured. Levels of CEC were significantly decreased to 280 cells/ml at 18 months post treatment compared to 1560 cells/ml at baseline (**Figure 8.6**).

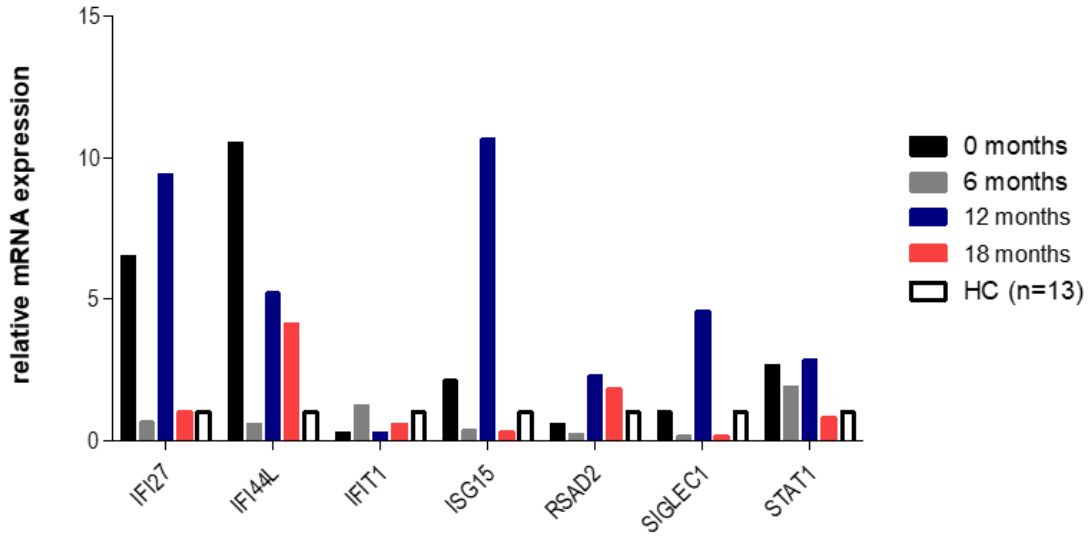

Figure 8.4. Interferon (IFN) type I related gene expression and signal transducer activator transcription (STAT)1 levels in the peripheral blood of a child with JDM treated with baricitinib: at baseline, 6 months, 12 months and 18 months post treatment. Both IFN type I related gene expression and STAT1 were decreased 6 and 18 months after starting baricitinib, while levels were increased at 12 months during a disease flare due to poor compliance.

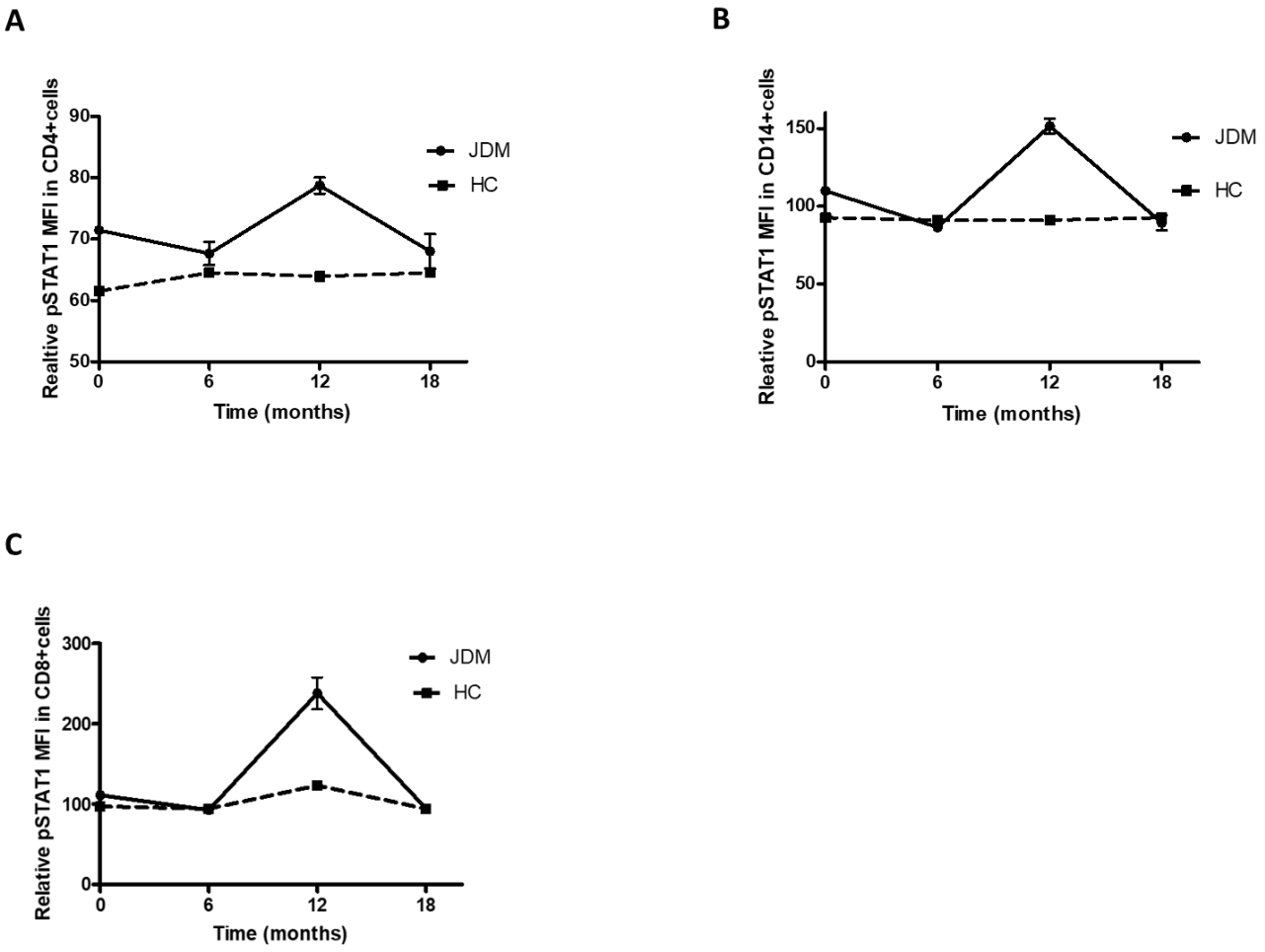

Figure 8.5. Signal transducer activator transcription (STAT) 1 phosphorylation in CD4+ (A), CD14+ (B) and CD8+ cells (C) in a child with Juvenile dermatomyositis (JDM) treated with baricitinib at the time of initiation of treatment (time 0), 6 months, 12 months and 18 months later (A-C) compared to healthy controls (HC). *STAT1 phosphorylation in all cell types decreased 6 months and 18 months post treatment while an increase was observed at 12 months' time during a disease flare when baricitinib was stopped due to compliance issues.*

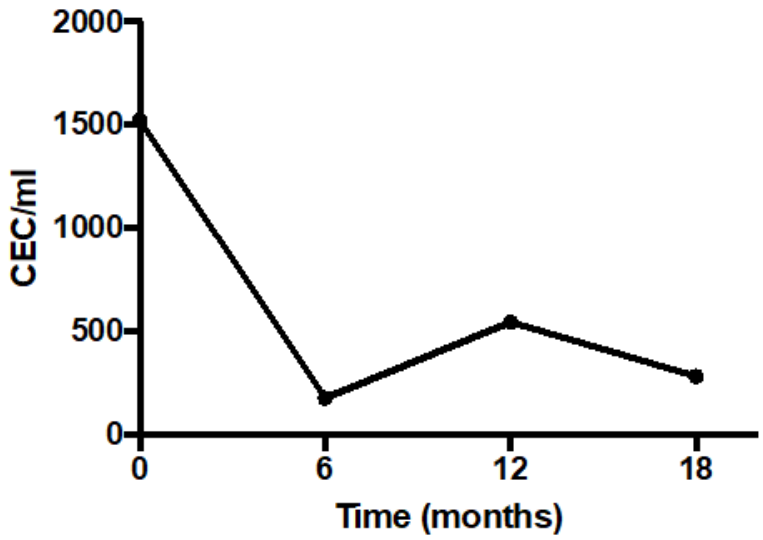

Figure 8.6. Circulating endothelial cells (CEC) in a child with Juvenile dermatomyositis (JDM) treated with baricitinib at time of initiation of treatment (time 0), 6 months, 12 months and 18 months later. *CEC decreased significantly at 6 months and 18 months of baricitinib treatment compared to the baseline. An increase in CEC levels was observed at 12 months when stop of baricitinib due to poor compliance was related with disease flare.*

8.5 Discussion

In this study, baricitinib, a JAK 1/2 inhibitor, was efficacious in a child with JDM with severe refractory skin disease. In keeping with the clinical response, there was improvement in the IFN stimulated gene expression profile in peripheral blood, providing proof of principle that JAK inhibitors may represent a more targeted and novel treatment for JDM. Additionally, levels of CEC, a biomarker of endothelial injury, significantly decreased on baricitinib treatment suggesting an efficacy of JAK inhibition on the vasculopathy of JDM. The fact that

there was both a clinical flare of symptoms but also an upregulation of the IFN stimulated gene expression when all medication was stopped, with a later improvement when treatment was re-introduced further supports those findings. Thus, the observed effects of baricitinib in immune pathways are likely to play key roles in modifying clinical responses and vasculopathy.

Although the immune mechanisms underlying JDM have not been fully elucidated, studies suggest that interferons are central to the pathogenesis of JDM and important drivers of the associated vasculopathy (Bilgic *et al.*, 2009). A number of studies have demonstrated the presence of IFN I in the blood, skin and muscle of patients with DM which also have been found to correlate with disease severity and activity (Greenberg *et al.*, 2005; Baechler *et al.*, 2007; Walsh *et al.*, 2007; Niewold *et al.*, 2009; Salajegheh *et al.*, 2010; Cappelletti *et al.*, 2011; Liao *et al.*, 2011; Suarez-Calvet *et al.*, 2014; Allenbach *et al.*, 2016; Uruha *et al.*, 2017). Recently, Ladislau *et al.* demonstrated a causative effect of type I IFN pathway activation to muscle atrophy and endothelial injury observed in DM (Ladislau *et al.*, 2018).

While treatments for JDM have improved over the last years, current therapeutic regimes still lack specificity, and in severe cases fail to control disease activity; moreover, these treatments also cause significant toxicity. More targeted treatments aimed at immune pathways like the type I IFN pathway that may be contributing to disease pathogenesis is an active area of research, and an unmet need. There are several steps in the type I IFN pathway that could

theoretically be potential therapeutic targets. Downregulation of type I IFN cytokines could be a potential approach, but since the exact type I IFNs involved in the pathogenesis of JDM are not yet known, this approach may prove challenging. Blockade of IFN receptors requires experimental medication only available as intravenous administration, a problematic issue in regard to the paediatric population. Oral JAK inhibitors that block the JAK/STAT pathway are therefore a more applicable approach. They have been proven to be efficacious in several autoimmune diseases with the interest focusing in SLE in which type I IFN pathway seems to also play a significant pathogenetic role (Baker and Isaacs, 2018; Wallace *et al.*, 2018). JAK inhibitors have also been shown to be effective in monogenic IFN-mediated autoinflammatory diseases (Fremond *et al.*, 2016; Sanchez *et al.*, 2018). Previous case reports and case series have demonstrated the efficacy of JAK inhibitors in DM patients (Hornung *et al.*, 2014; Kurtzman *et al.*, 2016; Paik and Christopher-Stine, 2017; Allenbach *et al.*, 2018; Kurasawa *et al.*, 2018; Ladislau *et al.*, 2018).

In the case reported in this chapter, no significant side effects were observed, notably absence of cytopenia or infections with herpes zoster, BK or JC viruses, all previously associated with JAK inhibitors (Taylor *et al.*, 2017). This is in keeping with the favorable outcome of the only other previously published report of ruxolitinib use in a child with JDM (Aeschlimann *et al.*, 2018). Moreover, baricitinib did not impair growth, a theoretical concern raised in view of the fact that growth hormone receptors transduce their signal through the JAK2/STAT4 pathway

(Carter-Su *et al.*, 2016). Again, this is in keeping with the Aeschlimann *et al*. report (Aeschlimann *et al.*, 2018).

My study has several limitations. Although, there was a significant improvement in clinical symptoms, the studied patient never achieved full clinical remission; and CEC did not completely return to healthy control levels. It is important to highlight that treatment with baricitinib was given after 8 years of active disease, and after failure of numerous treatments; thus, it was difficult to assess how many of the observed skin changes were representing damage and were not reversible. Additionally, as it is well known JAK inhibitors also block the signaling of other cytokines like IL-6, IL-12 and IL-23 that could also play a role in JDM pathogenesis. So, the observed effect could be the result not only of the type I IFN blockade but also of the blockade of these cytokines; thus, it is not possible to surmise that all the therapeutic benefit was derived solely from blockade of type I IFN.

In conclusion, this is the first report of treatment of JDM with baricitinib with significant clinical efficacy, and improvement in immune dysregulation and endothelial signal indicative of vasculopathy. This case represented one of the most treatment refractory patients from the large JDCBS cohort, where treatment with baricitinib was given 8 years after disease onset and after almost all available therapeutic options had failed. Thus, whilst only a single case, these preliminary observations suggest that JAK inhibition may be an effective treatment for JDM, and could warrant study of use earlier in the disease course.

9 General discussion and future directions

9.1 Summary: the vasculopathy of Juvenile dermatomyositis

IIM represent a group of systemic autoimmune diseases, characterised mainly by myositis and skin involvement, with JDM being the commonest subgroup in childhood cases (Meyer *et al.*, 2015). Studies of the affected tissues have demonstrated characteristic vascular alterations as a predominant feature of the disease, thus early descriptions recognise JDM as a systemic angiopathy of the childhood (Whitaker and Engel, 1972). The histological and capillaroscopic features of JDM vasculopathy include decrease in the number of small vessels, dilation and/or occlusion of capillaries, and capillary drop-out with different vascular changes observed at different times during the disease course. The exact pathophysiology of JDM vasculopathy remains unclear, however, and the detailed molecular mechanisms causing these vascular changes still remain largely elusive.

The degree and duration of vascular involvement has been associated with more severe and recalcitrant disease and the development of significant organ involvement leading to potentially life-threatening complications (Gitiaux *et al.*, 2016). Early detection and monitoring of vasculopathic features and early and appropriate therapeutic interventions are key components to a better outcome. Currently, the detection and monitoring of JDM vasculopathy over the disease course remains challenging. Therefore, there is increasing need for the development of new, specific, non-invasive biomarkers of JDM vasculopathy that will promote the better understanding of disease pathogenesis, will allow more accurate

prediction of disease outcome, will contribute into the development of new more targeted therapeutic regimes and will consist a tool for better monitoring of disease activity overtime. On the background of these challenges, this thesis investigated novel biomarkers of endothelial dysfunction and thrombotic propensity, while attempting to understand the role of endothelial injury and hypercoagulability in JDM pathogenesis.

A number of endothelial derived components that allow detection and monitoring of endothelial injury and damage has been studied in this thesis. In chapter 3, I began by exploring if CEC could serve as a biomarker that could track endothelial injury in children with JDM and found that children with active JDM had increased numbers of CEC compared with children with inactive disease and healthy control. Children with histopathological (vascular domain of muscle biopsy score) or capillaroscopic (abnormal nailfold capillaries) evidence of vasculopathy had higher CEC levels. In the prospective part of this study, CEC levels changed respectively only when there was a change into disease activity (from active to inactive and vice versa) suggesting that CEC could serve as a marker able not only to detect but also to monitor vascular injury over time in JDM patients. Interestingly, there was evidence of ongoing endothelial injury and damage in children with JDM even when they were considered to have clinically inactive disease irrespective of treatment suggesting that CEC could further help in identifying cases resistant to current treatment and guiding a stratified therapeutic approach to the treatment of each patient.

In chapter 4, I then explored the inflammatory protein profile associated with endothelial activation and dysfunction in children with JDM in order to identify biomarkers for monitoring disease activity relating to the vasculopathy of JDM. A number of soluble adhesion molecules and proteins related with endothelial activation were found to be significantly increased in children with JDM with galectin-9 and TNFRII demonstrating the best ability to discriminate between patients with active disease and patients with inactive disease, even under immunosuppression treatment. To discriminate between active and inactive disease in the studied cohort, cut-off values were then calculated for best performed markers. Galectin-9 levels strongly correlated with CEC levels. As type I IFNs are widely recognised to play a significant role in disease pathogenesis, plasma levels of IFN-α and IFN-β were also measured and found to be increased in children with active JDM, while for the first-time levels of IFN-λ1 were studied with interesting results suggesting a possible role for this IFN in JDM pathogenesis. When considering MSA subgroups, children with anti-MDA5 positive antibodies tended to have higher levels of type I IFNs and galectin-9 compared to other subgroups; but these results did not reach statistical significance. A much bigger study would be needed to confirm or refute any immunophenotype subgroupings associated with different MSA profiles and thus no conclusions can be drawn regarding that observation from my results.

In chapter 5, the MP profile associated with endothelial and cellular activation of children with JDM was investigated. Children with active JDM were found to have higher numbers of total AnV+, platelet, monocyte and B-cell (but not T-cell) derived MP. When I looked into the

MP profile of the 25 prospectively studied patients, similarly to CEC, circulating MP levels changed only when there was a change in disease activity status between the two studied time points suggesting that MP could serve as a biomarker for monitoring disease activity or predicting disease flares in children with JDM. I then examined if MP levels correlated with the other described biomarkers of endothelial injury, and I found that MP strongly correlated with both CEC and Galectin-9 levels.

In this thesis, it has been demonstrated that children with JDM were characterised by ongoing endothelial injury (as described in chapters 3, 4, and 5), had enhanced inflammatory profile (as described in chapter 4), and increased circulating platelet and monocyte derived MP expressing TF, with strong prothrombotic properties (as described in chapter 5). Thus in chapter 6, I further explored the prothrombotic state of JDM patients with measurement of plasma TGA, a method that can dissect the various stages of hypercoagulability (Saibeni *et al.*, 2010; Tripodi *et al.*, 2011; Solfietti *et al.*, 2016). Interestingly, children with both active and inactive disease were characterised by enhanced thrombin generation compared to healthy controls. ETP was then found to strongly correlate with levels of total AnV+ MP and TF+ monocyte derived MP, suggesting that MP could have an important prothrombotic role in the pathogenesis of JDM vasculopathy, and may provide a direct link between inflammation, thrombin generation, and vasculopathy. Based on these findings, a new possible therapeutic target for JDM patients can be considered; anti-thrombin agents, especially for patients with severe vasculopathic features. Dabigatran etexilate, a direct thrombin inhibitor, had anti-inflammatory and antifibrotic activity in a murine model of ILD (Bogatkevich *et al.*, 2011) and

its safety has been investigated in an-open-label phase 1 study in patients with systemic sclerosis associated ILD (Khanna *et al.*, 2019).

In chapter 7, I explored if the JDM vasculopathy in concert with traditional cardiovascular risk factors contributed to increased arterial stiffness in children with JDM. To investigate, whether children with JDM had findings of early atherosclerosis, PWV was performed in children with JDM, a surrogate marker or early atherosclerosis, compared to age-matched healthy controls. Interestingly, although no difference was identified in regard to carotid-femoral PWV, children with JDM were found to have increased carotid-radial PWV compared to healthy controls. As discussed in chapter 7, carotid-radial PWV reflects the state of medium-sized muscular arteries and whether increased stiffness of these arteries truly indicates generalised early atherosclerosis needs to be further elucidated.

Finally, based on the above-mentioned results, the effectiveness of baricitinib, a JAK 1/2 inhibitor was investigated in a child with recalcitrant skin disease (that had failed all currently available treatments) in chapter 8. The treated case had evidence of ongoing endothelial injury as demonstrated by increased CEC and abnormal IFN signal as demonstrated by upregulated IFN related gene expression despite conventional treatment. Introduction of baricitinib resulted in significant clinical improvement with the observed clinical changes reflecting also on CEC levels and associated with downregulation of IFN related gene expression.

To summarise, from this thesis different steps could be identified that could be used in the future to improve the care of patients with JDM:

(i) New and existing biomarkers, for use in clinical practise, of vascular injury and thrombosis in JDM and guide therapeutic management. These biomarkers may also be relevant for identifying disease severity risk in patients (children and adults) suffering from other inflammatory myositides and/or other systemic inflammatory diseases with a vasculopathic component.

(ii) A new insight into the pathogenesis of JDM has been provided by studying endothelial injury and a novel thrombotic propensity that could lead to small vessel intravascular thrombosis and occlusive vasculopathy.

(iii) By combining (i) and (ii) a scientific rationale for therapeutic stratification of individual patients could be provided and inform the use of entirely novel targeted modes of therapy in addition to instituting formal therapeutic lifestyle interventions to reduce this risk of accelerated cardiovascular morbidity before these patients progress into adulthood. For example, a stratification strategy based on these results could be:

a) Patients with persistent endothelial injury (increased CEC and/or EMP) and/or abnormal interferon signal (increased Galectin-9, MCP1, IP10 and/or IFNs) may benefit from JAK inhibitors which act directly downstream of interferon activation (Liu *et al.*, 2012). JDM patients with persistent endothelial injury could be ideal candidates to stratify for this newer treatment.

b) **Patients with evidence of pathological thrombin generation** (enhanced thrombin generation) might benefit from antithrombotic therapy, specifically consideration of directly targeting pathological thrombin generation using oral anti-thrombin agents (Lee and Ansell, 2011).

c) Lastly, if findings of structural arterial changes indicative of premature atherosclerosis in JDM are confirmed, this may be offset by using the new therapeutic approaches mentioned above, and also by instituting formal therapeutic lifestyle interventions to reduce this risk of accelerated cardiovascular morbidity before these patients progress into adulthood.

As already discussed in each of the chapters of this thesis, there are several limitations and areas that need to be further studied to confirm above presented data and to allow extrapolation of findings into clinical practise. To be able to address unanswered questions and to take this study forwards, the following future work is suggested.

9.2 Biomarker specificity for idiopathic inflammatory myopathies of childhood

The diagnosis of JDM can be straightforward in the presence of pathognomonic skin rashes and proximal muscle weakness. However, in the absence of characteristic skin rashes the diagnosis can be challenging. Causes of non-inflammatory myopathies include metabolic myopathies, muscular dystrophies and disorders of denervation or neuropathy. In those

cases, muscle biopsy is suggested to help the differential diagnosis. Muscle biopsy is, however, an invasive procedure and may not be available in specific paediatric centres. CEC and MP may be able to differentiate inflammatory from non-inflammatory myopathies and study of these biomarkers in the above-mentioned conditions in comparison to IIM may help define specificity of the findings described in my thesis.

9.3 Ability of biomarkers to predict disease outcome and response to treatment

My thesis has demonstrated that the studied biomarkers may be able to predict long-term outcomes and response to treatment, especially in regards to the vasculopathic features of JDM. Moreover, specific cut-offs have been proposed for the better discrimination of patients with active disease and patients with inactive disease. A prospective study is now needed to confirm these cut-offs. It would be also of great interest to observe whether these biomarkers reflect response to treatment, and may even predict disease flares. For example, if CEC, MP and galectin-9 levels are increased well in advance of a disease flare becoming clinically obvious, that may help to guide clinical decisions about changes in treatment. Moreover, combination of these novel biomarkers with more conventional JDM indices (e.g. capillaroscopy findings, muscle biopsy findings, MSA) may allow the development of a predictive model. Finally, these biomarkers may be used to guide clinical decisions on stopping treatment, and provide risk models to predict likelihood of flare after stopping treatment.

9.4 Study of biomarkers in relation to specific MSA subgroups and specific clinical manifestations

As discussed, IIM represent a group of highly heterogeneous diseases. In recent years, it has become evident that IIM patients can be further grouped based on the presence of specific MSA which are associated with different clinical phenotypes. Different MSA subgroups may also be associated with different pathogenic mechanisms. For example, it has now become evident that patients with anti-MDA5 antibodies (also known as interferon induced with helicase C domain, IFIH1, a classic type 1 interferon inducible protein) have a strong type I IFN signature, also related with significant lung involvement (Greenberg, 2010). It would be thus of great interest to examine whether these patients have increased levels of CEC, MP and galectin-9 and whether levels of these biomarkers are related with development of ILD.

9.5 Assessment of the protein content of MP and possible implications of MP in the pathogenesis of calcinosis

MP are recently recognized players of intercellular communication as they are able to transfer proteins, peptides, microRNA, mRNA and DNA between different cells without direct contact (Hugel *et al.*, 2005). Trying to further elucidate the role of MP into disease pathogenesis and their role in severe extra-muscular manifestations, the protein content of MP should be ascertained using proteomics. One limitation of this assay is that it requires large numbers of

MPs, limiting its utility in studies that involves children. Thus, alternative techniques should also be tested like flow cytometry (van der Pol *et al.*, 2010).

Several proteins are known to be implicated into the development of soft tissue calcification. A total of 30-35 % of children with JDM will develop calcinosis during the disease course (Bowyer *et al.*, 1986). Bone morphogenic protein (BMP) 2 is known to cause osteogenic differentiation of muscle cells and is produced by endothelial cells (Dalfino *et al.*, 2010). A recent study demonstrated that endothelial MP released upon cell damage are rich in BMP-2 and calcium and can induce calcification through osteogenic differentiation of vascular smooth muscle cells (Buendia *et al.*, 2015). Other proteins suggestive to play a key role into the pathogenesis of calcification is osteopontin, fetuin-A, other bone morphogenic proteins, matrix-gla-protein and osteoprotegin (Marhaug *et al.*, 2008; Scialla *et al.*, 2014). Thus, the role of these proteins in MP and development of calcinosis should be further investigated.

9.6 Further assessment of structural arterial changes in order to explore the presence of accelerated atherosclerosis in children with JDM

The present study has so far provided some evidence that children with JDM may have increased peripheral arterial stiffness as demonstrated by the increased carotid-radial PWV in relation to age slope. To further investigate that hypothesis, other surrogate markers of arterial stiffness and accelerated atherosclerosis should be used such as CIMT. Moreover, Endo-pat Stress Test, a non-invasive assessment of endothelial dysfunction, can be used in

order to shed light upon the state of the small vessels in children with JDM. Any potential future role of Endo-pat Stress testing as a novel non-invasive biomarker of JDM vasculopathy and/or atherosclerosis should be further investigated by relating any changes detected in this index to clinical disease activity; other markers of endothelial injury/systemic inflammation; and structural arterial changes. Stepwise multivariable linear regression could then be used to assess the relationship between PWV, CIMT and Endo-pat Stress test (dependent variables) and predictor variables, including each of the conventional cardiovascular risk factors, disease activity status, time from diagnosis, treatment, presence of calcinosis and each research parameter listed above. Inclusion of 150 subjects would permit up to 10-15 predictors to be included in the final model of cardiovascular health injury in JDM.

9.7 Final conclusion

In conclusion, this thesis demonstrated that there is increased endothelial injury in children with active JDM associated with high levels of CEC. In addition, there is increased endothelial, platelet, monocyte and B-cell membrane vesiculation resulting in increased plasma levels of circulating MP with propensity to drive thrombin generation and hence occlusive vasculopathy. Finally, a signal of increased arterial stiffness in paediatric patients with JDM has been demonstrated suggesting that premature atherosclerosis may develop as a consequence of JDM and its related vasculopathy. These novel non-invasive biomarkers relating to the vasculopathy of JDM can detect and monitor endothelial injury relating to subclinical disease activity in JDM over time and facilitate development of stratified treatment approaches to reduce long-term adverse outcomes for these children.

Appendix 1.

Muscle biopsy scoring tool in Juvenile Dermatomyositis

DOMAIN	Score		Definitions and Instructions
INFLAMMATORY DOMAIN			
CD3+ endomysial infiltration	0, 1, 2	☐	For each of endomysial, perimysial, perivascular distributions, score for CD3+ infiltrating cells as follows: if none, or <4 cells in ×20 field = score 0; if >4 cells in a ×20 field and/or 1 cluster (where a cluster is approx 10 cells or more) = score 1; if >2 clusters in whole biopsy, and/or diffusely infiltrating cells (i.e., >20 cells in a ×20 field) = score 2
CD3+ perimysial infiltration	0, 1, 2	☐	
CD3+ perivascular infiltration	0, 1, 2	☐	
CD68+ endomysial infiltration	0, 1, 2	☐	For each of endomysial, perimysial, perivascular distributions, score for CD68+ infiltrating cells as follows: if none, or <4 cells in ×20 field = score 0; if >4 cells in a ×20 field and/or 1 cluster (where a cluster is approx 10 cells or more) = score 1; if >2 clusters in whole biopsy, and/or diffusely infiltrating cells (ie, >20 cells in a 20× field) = score 2
CD68+ perimysial infiltration	0, 1, 2	☐	
CD68+ perivascular infiltration	0, 1, 2	☐	
VASCULAR DOMAIN			
Capillary dropout (Using stain for CD31)	N or Y	☐	Obvious and marked decrease in the density of capillary network, not restricted to areas of perifascicular atrophy. Absence = N, presence = Y
Arterial abnormality	N or Y	☐	Mural thickening and/or endothelial swelling and/or transmural inflammation in arteries/arterioles. Absence = N, presence = Y
Infarction	N or Y	☐	Well demarcated regional loss of muscle fiber nuclei and loss of normal cytoarchitecture. Absence = N, presence = Y
MUSCLE FIBER DOMAIN			
MHC Class I over- expression	N or Y	☐	Presence of MHC class I staining on or in muscle fibers. Absence = N, presence = Y
Perifascicular atrophy	0, 1, 2	☐	Affecting >6 fibers out of 10 along one edge of a fasciculus, not exclusive to type IIb fibers. Absent = score 0. Present in one or 2 fascicles = score 1. Present in 3 or more fascicles = score 2
Neonatal myosin (using stain for neonatal myosin)	0 or 1	☐	Less than 6 positive fibers in a ×20 field = score 0; >6 positive fibers in a ×x20 field = score 1
Fiber atrophy: non perifascicular	N or Y	☐	Fiber atrophy: non-perifascicular (outside normal variation for age). Absence = N, presence = Y.
Regeneration/ Degeneration /Necrosis: peri-fascicular	0, 1, 2	☐	Includes: focal basophilia within a fiber, vacuolation, myofibrillar rarefaction, and/or pallor, myophagocytosis, acid phosphatase positive fibers. For each of perifasciular and non-perifascicular, score as follows. None = score 0. If any of the features in 1 or 2 fasciculi = score 1. If any of the features in 3 or more fasciculi = score 2.
Regeneration/ Degeneration /Necrosis: non-peri-fascicular	0, 1, 2	☐	
Internal myonuclei in non-basophilic otherwise normal fibers	0 or 1	☐	Internal myonuclei in non-basophilic cells (in otherwise normal fibers) in 1 or more fasciculi excluding myotendinous junctions. If <3% fibers = score 0. If >3% fibers = score 1
CONNECTIVE TISSUE DOMAIN			
Any endomysial fibrosis	N or Y	☐	For fibrosis in each of endomysial and perimysial distributions, score as follows: Absence = score N. Presence of any = score Y
Any perimysial fibrosis	N or Y	☐	

VISUAL ANALOG SCALE FOR OVERALL IMPRESSION OF ABNORMALITY

0 _____ **10**

no abnormality **much abnormality**

Appendix 2.

Childhood Myositis Assessment score (CMAS)

Childhood Myositis Assessment Score (CMAS)

Patient Reference Number:_____ **Date of visit:**_____

1. Head Elevation (neck flexion): **Item Score:**_____
0 = unable
1 = 1-9 seconds
2 = 10-29 seconds
3 = 30-59 seconds
4 = 60-119 seconds
5 = >2 minutes
No. of seconds:.....

2. Leg raise/touch object: **Item Score:**_____
0 = unable to lift leg off table
1 = able to clear table but cannot touch object
2 = able to lift leg high enough to touch object

3. Straight leg lift/duration: **Item Score:**_____
0 = unable
1 = 1-9 seconds
2 = 10-29 seconds
3 = 30-59 seconds
4 = 60-119 seconds
5 = >2 minutes
No. of seconds:.....

4. Supine to prone: **Item Score:**_____
0 = unable. Has difficulty even turning onto side; able to pull arms under torso only slightly or not at all
1 = turns onto side fairly easily; but cannot fully free arms and is not able to fully assume a prone position
2 = Easily turns onto side; has some difficulty freeing arms, but fully frees them and fully assumes a prone position
3 = easily turns over, free arms with no difficulty

5. Sit ups: **Item Score:**_____
For each type of sit-up enter either "0" (unable) or "1" (able). Then enter the total sub score (maximum possible item score 6).
Hands on thighs, with counterbalance -
Hands across chest, with counterbalance -
Hands behind head, with counterbalance -
Hands on thighs, without counterbalance -
Hands across chest, without counterbalance -
Hands behind head, without counterbalance -

6. Supine to sit: **Item Score:**_____
0 = unable to self.
1 = much difficulty. Very slow, struggles greatly, barely makes it. Almost unable
2 = some difficulty. Able, but is somewhat slow, struggles some.
3 = No difficulty

7. Arm raise/straighten: **Item Score:**_____
0 = cannot raise wrists
1 = can raise wrists at least up to the level of the acromioclavicular joint but not above top of head
2 = can raise wrists above top of head but cannot raise arms straight above head so that elbows are in full extension
3 = can raise arms straight above head so that elbows are in full extension.

8. Arm raise/duration: **Item Score:**_____
Can maintain wrists above top of head for:
0 = unable
1 = 1-9 seconds
2 = 10-29 seconds
3 = 30-59 seconds
4 = >60 seconds
No. of seconds:.....

Total Score (Max out of 52):_____

Lovell et al, 1999 and Huber et al, 2004

9. Floor sit: **Item Score:**_____
Going from a standing position to a sitting position on the floor
0 = unable. Afraid to even try. Even if allowed to use a chair for support. Child fears that he/she will collapse, fall into a sit or self-harm
1 = much difficulty. Able, but needs to hold onto chair for support during descent (unable to unwilling to try if not able to use a chair for support)
2 = some difficulty. Can go from stand to sit without using a chair for support but has at least some difficulty during descent. Descends somewhat slowly and/or apprehensively; may not have full control or balance as manoeuvres into a sit
3 = No difficulty. Requires no compensatory manoeuvring

10. All-fours manoeuvre: **Item Score:**_____
0 = unable to go from a prone to an all-fours position
1 = barely able to assume and maintain an all-fours position
2 = can maintain all-fours position with straight back and head raised (so as to look straight ahead). But cannot crawl forward
3 = Can maintain all fours, look straight ahead and crawl forward
4 = maintains balance while lifting and extending leg

11. Floor rise: **Item Score:**_____
Going from a kneeling position on the floor to a standing position
0 = unable, even if allowed to use a chair for support
1 = much difficulty. Able, but needs to use a chair for support. Unable if not allowed to use a chair
2 = Moderate difficulty. Able to get up without a chair for support but needs to place on or both hands on thighs/knees or floor. Unable without using hands.
3 = mild difficulty. Does not need to place hands on knees, thighs or floor but has at least some difficulty during assent.
4 = no difficulty

12. Chair rises: **Item Score:**_____
0 = unable to rise form chair, even if allowed to place hands on sides of chair
1 = Much difficulty. Able but needs to place hands on side of seat. Unable if not allowed to place hands on knees/thighs
2 = moderate difficulty. Able but needs to place hands on knees/thighs. Does not need to place hands on side of seat
3 = mild difficulty. Able; does not need to use hands at all, but has at least some difficulty
4 = no difficulty

13. Stool step: **Item Score:**_____
0 = unable
1 = much difficulty. Able but needs to place one hand on exam table or examiner's hand,
2 = some difficulty. Able; does not need use exam table for support but needs to use hands on knee/thigh
3. Able. Does not need to use exam table or hands on knee/thigh

14. Pick up: **Item Score:**_____
0 = able to bend over and pick up pencil on floor
1 = much difficulty. Able but relies heavily on support gained by placing hands on knees/thighs
2 = some difficulty. Needs to at least minimally and briefly place hands on knees/thighs for support and is somewhat slow
3 = No difficulty. No compensatory manoeuvre necessary.

Appendix 3.

Manual Muscle Testing (MMT)-8

MYOMETRY

Kendall Left	MMT * Left	Myometry Left	Date		Myometry Right	MMT * Right	Kendall Right
/10			NECK	Flexors			/10
				Extensors			
			BACK	Extensors			
			ABDOMINALS				
/10			SHOULDER	Abductors			/10
/10			ELBOW	Flexors			/10
/10			WRIST	Extensors			/10
			FINGER	Grip			
			HIP	Flexors			
/10				Extensors			/10
/10				Abductors			/10
			KNEE	Flexors			
				Extensors-90°			
/10				Extensors-0°			/10
/10			ANKLE	Dorsiflexors			/10
/80			TOTAL				/80

*** MMT**: 0=no muscle action, 1=flicker of muscle action, 2=muscle action with gravity counterbalance, 3=muscle action against gravity, 4=muscle action against gravity with some resistance, 5=full muscle strength, (9=not done)

STANDARD SCORE FOR Kendall MMT (0–10 SCALE)		
	FUNCTION OF THE MUSCLE	**0–10 SCALE**
No Movement		0
Test Movement	**MOVEMENT IN HORIZONTAL PLANE**	
	Moves through partial range of motion	1
	Moves through complete range of motion	2
	Moves to completion of range against resistance Or Moves to completion of range and holds against pressure Or	3
	ANTIGRAVITY POSITION	
	Moves through partial range of motion	
Test Position	Gradual release from test position	4
	Holds test position (no added pressure)	5
	Holds test position against slight pressure	6
	Holds test position against slight to moderate pressure	7
	Holds test position against moderate pressure	8
	Holds test position against moderate to strong pressure	9
	Holds test position against strong pressure	10

Version 3: Created December 2008

Appendix 4.

Childhood Health Assessment Questionnaire (CHAQ)

CHILDHOOD HEALTH ASSESSMENT QUESTIONNAIRE

In this section we are interested in learning how your child's pain affects his/her ability to function in daily life. Please feel free to add any comments on the back of this page. In the following questions, please tick the one response which best describes your child's usual activities OVER THE PAST WEEK. If most children at your child's age are not expected to do a certain activity, please mark it as "Not Applicable". For example, if your child has difficulty in doing a certain activity or is unable to do it because he/she is too young but not because he/she is RESTRICTED BY PAIN or ILLNESS, please mark it as "NOT Applicable".

	Without ANY Difficulty	With SOME Difficulty	With MUCH Difficulty	UNABLE To do	Not Applicable
DRESSING & PERSONAL CARE					
Is your child able to:					
- Dress, including tying shoelaces and doing buttons?	☐	☐	☐	☐	☐
- Shampoo his/her hair?	☐	☐	☐	☐	☐
- Remove socks?	☐	☐	☐	☐	☐
- Cut fingernails?	☐	☐	☐	☐	☐
GETTING UP					
Is your child able to:					
- Stand up from a low chair or floor?	☐	☐	☐	☐	☐
- Get in and out of bed or stand up in a cot?	☐	☐	☐	☐	☐
EATING					
Is your child able to:					
- Cut his/her own meat?	☐	☐	☐	☐	☐
- Lift up a cup or glass to mouth?	☐	☐	☐	☐	☐
- Open a new cereal box?	☐	☐	☐	☐	☐
WALKING					
Is your child able to:					
- Walk outside on flat ground?	☐	☐	☐	☐	☐

- Climb up five steps?	☐	☐	☐	☐	☐

* **Please tick any AIDS or DEVICES that your child usually uses for any of the above activities**:

- Walking stick	☐	- Devices used for dressing (button hook, zip pull, long-handled shoe horn, etc.)	☐
- Walking frame	☐	- Built up pencil or special utensils	☐
- Crutches	☐	- Special or built up chair	☐
- Wheelchair	☐	- Other (Specify:_____)	☐

*** Please tick any categories for which your child usually needs help from another person BECAUSE OF PAIN or ILLNESS:**

- Dressing and personal care	☐	- Eating	☐
- Getting up	☐	- Walking	☐

HYGIENE

Is your child able to:

- Wash and dry entire body?	☐	☐	☐	☐	☐
- Take a bath (get in and out of bath)?	☐	☐	☐	☐	☐
- Get on and off the toilet or potty?	☐	☐	☐	☐	☐
- Brush teeth?	☐	☐	☐	☐	☐
- Comb/brush hair?	☐	☐	☐	☐	☐

REACH

Is your child able to:

- Reach and get down a heavy object such as a large game or books from just above his/her head?	☐	☐	☐	☐	☐
- Bend down to pick up clothing or a piece of paper from the floor?	☐	☐	☐	☐	☐
- Pull on a jumper over his/her head?	☐	☐	☐	☐	☐
- Turn neck to look back over shoulder?	☐	☐	☐	☐	☐

GRIP					
Is your child able to:					
- Write or scribble with pen or pencil?	☐	☐	☐	☐	☐
- Open car doors?	☐	☐	☐	☐	☐
- Open jars which have been previously opened?	☐	☐	☐	☐	☐
- Turn taps on and off?	☐	☐	☐	☐	☐
- Push open a door when he/she has to turn a door knob?	☐	☐	☐	☐	☐
ACTIVITIES					
Is your child able to:					
- Run errands and shop?	☐	☐	☐	☐	☐
- Get in and out of a car or toy car or school bus?	☐	☐	☐	☐	☐
- Ride bike or tricycle?	☐	☐	☐	☐	☐
- Do household chores (e.g. wash dishes, take out rubbish, hoovering, gardening, make bed, clean room)?	☐	☐	☐	☐	☐
- Run and play?	☐	☐	☐	☐	☐

* **Please tick any AIDS or DEVICES that your child usually uses for any of the above activities:**

- Raised toilet seat	☐	- Bathrail	☐
- Bath seat	☐	- Long-handled appliances for reach	☐
- Jar opener (for jars previously opened)	☐	- Long-handled appliances in bathroom	☐

* Please tick any categories for which your child usually needs help from another person BECAUSE OF PAIN:

- Hygiene	☐	- Gripping and opening things	☐
- Reach	☐	- Errands and chores	☐

Appendix 5.

Test characteristics of total Annexin V positive microparticles for identification of children with active Juvenile dermatomyositis (JDM).

Total AnV+MP	Sensitivity (95% C.I.)	Specificity (95% C.I.)	+LR	-LR
>=268.832	100.0 (100.0-100.0)	0.0 (0.0- 0.0)	1	
> 268.832	98.7 (92.9- 99.8)	0.0 (0.0- 0.0)	0.99	
> 504.06	98.7 (92.9- 99.8)	2.7 (0.5-14.2)	1.01	0.48
> 1881.824	98.7 (92.9- 99.8)	5.4 (0.8- 18.2)	1.04	0.24
> 5242.2241	98.7 (92.9- 99.8)	8.1 (1.8- 21.9)	1.07	0.16
> 7185.6719	98.7 (92.9- 99.8)	10.8 (3.1- 25.4)	1.11	0.12
> 11044.6396	98.7 (92.9- 99.8)	13.5 (4.6- 28.8)	1.14	0.1
> 17233.6699	98.7 (92.9- 99.8)	16.2 (6.2- 32.0)	1.18	0.08
> 18518.6309	98.7 (92.9- 99.8)	18.9 (8.0- 35.2)	1.22	0.07
> 24750.6504	98.7 (92.9- 99.8)	21.6 (9.9- 38.2)	1.26	0.06
> 25282.9199	98.7 (92.9- 99.8)	24.3 (11.8- 41.2)	1.3	0.05
> 42341.0391	98.7 (92.9- 99.8)	27.0 (13.8- 44.1)	1.35	0.05
> 46597.5508	97.4 (90.9- 99.6)	27.0 (13.8- 44.1)	1.33	0.1

> 54474.7109	97.4 (90.9- 99.6)	29.7 (15.9- 47.0)	1.39	0.09
> 56060.1992	97.4 (90.9- 99.6)	32.4 (18.0- 49.8)	1.44	0.08
> 59017	97.4 (90.9- 99.6)	35.1 (20.2- 52.5)	1.5	0.07
> 61209.0586	97.4 (90.9- 99.6)	37.8 (22.5- 55.2)	1.57	0.07
> 65424.5586	97.4 (90.9- 99.6)	40.5 (24.8- 57.9)	1.64	0.06
> 65551.0234	97.4 (90.9- 99.6)	43.2 (27.1- 60.5)	1.72	0.06
> 71912.5625	96.1 (89.0- 99.1)	43.2 (27.1- 60.5)	1.69	0.09
> 72920.6797	96.1 (89.0- 99.1)	45.9 (29.5- 63.1)	1.78	0.08
> 74003.4766	96.1 (89.0- 99.1)	48.6 (31.9- 65.6)	1.87	0.08
> 75879	94.8 (87.2- 98.5)	48.6 (31.9- 65.6)	1.85	0.11
> 81266.5313	94.8 (87.2- 98.5)	51.4 (34.4- 68.1)	1.95	0.1
> 89139.2031	94.8 (87.2- 98.5)	54.1 (36.9- 70.5)	2.06	0.1
> 90393.0313	93.5 (85.5- 97.8)	54.1 (36.9- 70.5)	2.04	0.12
> 91054.7969	92.2 (83.8- 97.1)	54.1 (36.9- 70.5)	2.01	0.14
> 91924.7969	90.9 (82.2- 96.3)	54.1 (36.9- 70.5)	1.98	0.17
> 93369.3828	90.9 (82.2- 96.3)	56.8 (39.5- 72.9)	2.1	0.16
> 97619.6172	89.6 (80.5- 95.4)	56.8 (39.5- 72.9)	2.07	0.18
> 98979.9375	89.6 (80.5- 95.4)	59.5 (42.1- 75.2)	2.21	0.17
> 103682.101	89.6 (80.5- 95.4)	62.2 (44.8- 77.5)	2.37	0.17
> 104713	89.6 (80.5- 95.4)	64.9 (47.5- 79.8)	2.55	0.16

> 106748.703	88.3 (79.0- 94.5)	64.9 (47.5- 79.8)	2.51	0.18
> 107618.703	87.0 (77.4- 93.6)	64.9 (47.5- 79.8)	2.48	0.2
> 112469.5	87.0 (77.4- 93.6)	67.6 (50.2- 82.0)	2.68	0.19
> 113186.203	87.0 (77.4- 93.6)	70.3 (53.0- 84.1)	2.93	0.18
> 121524	85.7 (75.9- 92.6)	70.3 (53.0- 84.1)	2.88	0.2
> 123102.703*	85.7 (75.9- 92.	73.0 (55.9- 86.2)	3.17	0.2
> 142905.5	84.4 (74.4- 91.7)	73.0 (55.9- 86.2)	3.12	0.21
> 144378.796	83.1 (72.9- 90.7)	73.0 (55.9- 86.2)	3.08	0.23
> 149351.093	81.8 (71.4- 89.7)	73.0 (55.9- 86.2)	3.03	0.25
> 156816.593	80.5 (69.9- 88.7)	73.0 (55.9- 86.2)	2.98	0.27
> 167049	79.2 (68.5- 87.6)	73.0 (55.9- 86.2)	2.93	0.28
> 169700.203	77.9 (67.0- 86.6)	73.0 (55.9- 86.2)	2.88	0.3
> 179949.406	76.6 (65.6- 85.5)	73.0 (55.9- 86.2)	2.84	0.32
> 185562	75.3 (64.2- 84.4)	73.0 (55.9- 86.2)	2.79	0.34
> 186999.593	74.0 (62.8- 83.4)	73.0 (55.9- 86.2)	2.74	0.36
> 189516.593	74.0 (62.8- 83.4)	75.7 (58.8- 88.2)	3.04	0.34
> 190726.5	72.7 (61.4- 82.3)	75.7 (58.8- 88.2)	2.99	0.36
> 191005.093	71.4 (60.0- 81.1)	75.7 (58.8- 88.2)	2.94	0.38
> 191564.406	71.4 (60.0- 81.1)	78.4 (61.8- 90.1)	3.3	0.36
> 203018.5	71.4 (60.0- 81.1)	81.1 (64.8- 92.0)	3.78	0.35

> 204422.406	70.1 (58.6- 80.0)	81.1 (64.8- 92.0)	3.71	0.37
> 204924.703	68.8 (57.3- 78.9)	81.1 (64.8- 92.0)	3.64	0.38
> 207701.296	67.5 (55.9- 77.8)	81.1 (64.8- 92.0)	3.57	0.4
> 207966.296	66.2 (54.6- 76.6)	81.1 (64.8- 92.0)	3.5	0.42
> 217216.296	64.9 (53.2- 75.5)	81.1 (64.8- 92.0)	3.43	0.43
> 230691.5	63.6 (51.9- 74.3)	81.1 (64.8- 92.0)	3.36	0.45
> 246873.796	62.3 (50.6- 73.1)	81.1 (64.8- 92.0)	3.29	0.46
> 249677.703	61.0 (49.2- 72.0)	81.1 (64.8- 92.0)	3.23	0.48
> 262942.406	59.7 (47.9- 70.8)	81.1 (64.8- 92.0)	3.16	0.5
> 268948.906	59.7 (47.9- 70.8)	83.8 (68.0- 93.8)	3.68	0.48
> 277890.312	58.4 (46.6- 69.6)	83.8 (68.0- 93.8)	3.6	0.5
> 283556	57.1 (45.4- 68.4)	83.8 (68.0- 93.8)	3.52	0.51
> 284100.187	55.8 (44.1- 67.2)	83.8 (68.0- 93.8)	3.44	0.53
> 288396.687	54.5 (42.8- 65.9)	83.8 (68.0- 93.8)	3.36	0.54
> 289201.093	53.2 (41.5- 64.7)	83.8 (68.0- 93.8)	3.28	0.56
> 293857.187	51.9 (40.3- 63.5)	83.8 (68.0- 93.8)	3.2	0.57
> 298072.312	50.6 (39.0- 62.2)	83.8 (68.0- 93.8)	3.12	0.59
> 298967.812	50.6 (39.0- 62.2)	86.5 (71.2- 95.4)	3.75	0.57
> 300990.687	49.4 (37.8- 61.0)	86.5 (71.2- 95.4)	3.65	0.59
> 306244.5	48.1 (36.5- 59.7)	86.5 (71.2- 95.4)	3.56	0.6

> 308656.5	46.8 (35.3- 58.5)	86.5 (71.2- 95.4)	3.46	0.62
> 322766.812	45.5 (34.1- 57.2)	86.5 (71.2- 95.4)	3.36	0.63
> 327046.812	44.2 (32.8- 55.9)	86.5 (71.2- 95.4)	3.27	0.65
> 330807.093	42.9 (31.6- 54.6)	86.5 (71.2- 95.4)	3.17	0.66
> 335838.906	41.6 (30.4- 53.4)	86.5 (71.2- 95.4)	3.08	0.68
> 342087.812	40.3 (29.2- 52.1)	86.5 (71.2- 95.4)	2.98	0.69
> 390554.593	39.0 (28.0- 50.8)	86.5 (71.2- 95.4)	2.88	0.71
> 394781.593	39.0 (28.0- 50.8)	89.2 (74.6- 96.9)	3.6	0.68
> 400135.312	37.7 (26.9- 49.4)	89.2 (74.6- 96.9)	3.48	0.7
> 402867.406	36.4 (25.7- 48.1)	89.2 (74.6- 96.9)	3.36	0.71
> 407409.906	35.1 (24.5- 46.8)	89.2 (74.6- 96.9)	3.24	0.73
> 410589.687	33.8 (23.4- 45.4)	89.2 (74.6- 96.9)	3.12	0.74
> 418498.906	32.5 (22.2- 44.1)	89.2 (74.6- 96.9)	3	0.76
> 453096.5	32.5 (22.2- 44.1)	91.9 (78.1- 98.2)	4	0.73
> 465751.406	31.2 (21.1- 42.7)	91.9 (78.1- 98.2)	3.84	0.75
> 508546.093	29.9 (20.0- 41.4)	91.9 (78.1- 98.2)	3.68	0.76
> 520862	28.6 (18.9- 40.0)	91.9 (78.1- 98.2)	3.52	0.78
> 521597.906	27.3 (17.7- 38.6)	91.9 (78.1- 98.2)	3.36	0.79
> 542843.812	26.0 (16.6- 37.2)	91.9 (78.1- 98.2)	3.2	0.81
> 547745.187	24.7 (15.6- 35.8)	91.9 (78.1- 98.2)	3.04	0.82

> 621358.875	23.4 (14.5- 34.4)	91.9 (78.1- 98.2)	2.88	0.83
> 623423.625	22.1 (13.4- 33.0)	91.9 (78.1- 98.2)	2.72	0.85
> 687403.375	20.8 (12.4- 31.5)	91.9 (78.1- 98.2)	2.56	0.86
> 695979.125	19.5 (11.3- 30.1)	91.9 (78.1- 98.2)	2.4	0.88
> 707446.5	18.2 (10.3- 28.6)	91.9 (78.1- 98.2)	2.24	0.89
> 725909.125	16.9 (9.3- 27.1)	91.9 (78.1- 98.2)	2.08	0.9
> 727302.625	15.6 (8.3- 25.6)	91.9 (78.1- 98.2)	1.92	0.92
> 729281.5	14.3 (7.4- 24.1)	91.9 (78.1- 98.2)	1.76	0.93
> 947500	13.0 (6.4- 22.6)	91.9 (78.1- 98.2)	1.6	0.95
> 956059.875	11.7 (5.5- 21.0)	91.9 (78.1- 98.2)	1.44	0.96
> 1074320	10.4 (4.6- 19.5)	91.9 (78.1- 98.2)	1.28	0.98
> 1075061	9.1 (3.7- 17.8)	91.9 (78.1- 98.2)	1.12	0.99
> 1256162	7.8 (2.9- 16.2)	91.9 (78.1- 98.2)	0.96	1
> 1381667	7.8 (2.9- 16.2)	94.6 (81.8- 99.2)	1.44	0.97
> 1455191	7.8 (2.9- 16.2)	97.3 (85.8- 99.5)	2.88	0.95
> 1629923	6.5 (2.2- 14.5)	97.3 (85.8- 99.5)	2.4	0.96
> 1678191	5.2 (1.5- 12.8)	97.3 (85.8- 99.5)	1.92	0.97
> 1929153	5.2 (1.5- 12.8)	100.0 (100.0-100.0)		0.95
> 2037836	3.9 (0.9- 11.0)	100.0 (100.0-100.0)		0.96
> 2123095	2.6 (0.4- 9.1)	100.0 (100.0-100.0)		0.97

> 3453780	1.3 (0.2- 7.1)	100.0 (100.0-100.0)		0.99
> 7887903	0.0 (0.0- 0.0)	100.0 (100.0-100.0)		1

CI=confidence intervals; LR+ = Likelihood ratio for a positive test result; LR- = Likelihood ratio for a negative test result.

Appendix 6.

Test characteristics of plasma peak thrombin for identification of children with active Juvenile dermatomyositis (JDM).

Peak thrombin	Sensitivity (95% C.I.)	Specificity (95% C.I.)	+LR	-LR
≥ 38	100.0 (100.0-100.0)	0.0 (0.0- 0.0)	1	
> 38	100.0 (100.0-100.0)	2.4 (0.4- 12.9)	1.02	0
> 43.6	100.0 (100.0-100.0)	4.9 (0.7- 16.6)	1.05	0
> 52.3	100.0 (100.0-100.0)	7.3 (1.6- 19.9)	1.08	0
> 59.8156	98.6 (92.5- 99.8)	7.3 (1.6- 19.9)	1.06	0.19
> 61.4306	98.6 (92.5- 99.8)	9.8 (2.8- 23.1)	1.09	0.14
> 68.5	98.6 (92.5- 99.8)	12.2 (4.1- 26.2)	1.12	0.11
> 72.1589	97.2 (90.3- 99.6)	12.2 (4.1- 26.2)	1.11	0.23
> 74.1199	97.2 (90.3- 99.6)	14.6 (5.6- 29.2)	1.14	0.19
> 78.8	97.2 (90.3- 99.6)	17.1 (7.2- 32.1)	1.17	0.16

> 80.5	97.2 (90.3- 99.6)	19.5 (8.8- 34.9)	1.21	0.14
> 84.7328	97.2 (90.3- 99.6)	22.0 (10.6- 37.6)	1.25	0.13
> 86.4632	95.8 (88.3- 99.1)	22.0 (10.6- 37.6)	1.23	0.19
> 87.8475	95.8 (88.3- 99.1)	24.4 (12.4- 40.3)	1.27	0.17
> 92.9	95.8 (88.3- 99.1)	26.8 (14.2- 42.9)	1.31	0.16
> 94.7	94.4 (86.4- 98.4)	26.8 (14.2- 42.9)	1.29	0.21
> 96.6	94.4 (86.4- 98.4)	29.3 (16.1- 45.5)	1.34	0.19
> 97.6529	93.1 (84.5- 97.7)	29.3 (16.1- 45.5)	1.32	0.24
> 98.4604	93.1 (84.5- 97.7)	31.7 (18.1- 48.1)	1.36	0.22
> 102.3826	91.7 (82.7- 96.9)	31.7 (18.1- 48.1)	1.34	0.26
> 105.4972	90.3 (81.0- 96.0)	31.7 (18.1- 48.1)	1.32	0.31
> 105.8	88.9 (79.3- 95.1)	31.7 (18.1- 48.1)	1.3	0.35
> 106.9969	88.9 (79.3- 95.1)	34.1 (20.1- 50.6)	1.35	0.33
> 108.2	88.9 (79.3- 95.1)	36.6 (22.1- 53.1)	1.4	0.3
> 108.7272	87.5 (77.6- 94.1)	39.0 (24.2- 55.5)	1.43	0.32
> 109.5347	86.1 (75.9- 93.1)	39.0 (24.2- 55.5)	1.41	0.36
> 109.6501	84.7 (74.3- 92.1)	39.0 (24.2- 55.5)	1.39	0.39
> 110.2269	84.7 (74.3- 92.1)	41.5 (26.3- 57.9)	1.45	0.37
> 112.6494	83.3 (72.7- 91.1)	41.5 (26.3- 57.9)	1.42	0.4
> 113.4569	83.3 (72.7- 91.1)	43.9 (28.5- 60.2)	1.49	0.38

> 114.0337	81.9 (71.1- 90.0)	43.9 (28.5- 60.2)	1.46	0.41
> 115.8	80.6 (69.5- 88.9)	43.9 (28.5- 60.2)	1.44	0.44
> 118.8787	79.2 (68.0- 87.8)	43.9 (28.5- 60.2)	1.41	0.47
> 120.3784	79.2 (68.0- 87.8)	46.3 (30.7- 62.6)	1.48	0.45
> 121.1859	77.8 (66.4- 86.7)	46.3 (30.7- 62.6)	1.45	0.48
> 124.7619	77.8 (66.4- 86.7)	48.8 (32.9- 64.9)	1.52	0.46
> 125.3387	76.4 (64.9- 85.6)	48.8 (32.9- 64.9)	1.49	0.48
> 126.4923	75.0 (63.4- 84.5)	48.8 (32.9- 64.9)	1.46	0.51
> 127.4152	73.6 (61.9- 83.3)	48.8 (32.9- 64.9)	1.44	0.54
> 128.2227	73.6 (61.9- 83.3)	51.2 (35.1- 67.1)	1.51	0.52
> 128.5687	72.2 (60.4- 82.1)	51.2 (35.1- 67.1)	1.48	0.54
> 128.7	72.2 (60.4- 82.1)	53.7 (37.4- 69.3)	1.56	0.52
> 134.3366	72.2 (60.4- 82.1)	56.1 (39.8- 71.5)	1.65	0.5
> 135.6056	70.8 (58.9- 81.0)	58.5 (42.1- 73.7)	1.71	0.5
> 135.8	69.4 (57.5- 79.8)	58.5 (42.1- 73.7)	1.67	0.52
> 137.7974	68.1 (56.0- 78.6)	61.0 (44.5- 75.8)	1.74	0.52
> 138.7202	66.7 (54.6- 77.3)	61.0 (44.5- 75.8)	1.71	0.55
> 140.2199	66.7 (54.6- 77.3)	63.4 (46.9- 77.9)	1.82	0.53
> 140.7967	66.7 (54.6- 77.3)	65.9 (49.4- 79.9)	1.95	0.51
> 143.1038	65.3 (53.1- 76.1)	65.9 (49.4- 79.9)	1.91	0.53

> 144.8342	63.9 (51.7- 74.9)	65.9 (49.4- 79.9)	1.87	0.55
> 145.2956	62.5 (50.3- 73.6)	65.9 (49.4- 79.9)	1.83	0.57
> 147.7181	61.1 (48.9- 72.4)	65.9 (49.4- 79.9)	1.79	0.59
> 149.4	61.1 (48.9- 72.4)	68.3 (51.9- 81.9)	1.93	0.57
> 151.5249	61.1 (48.9- 72.4)	70.7 (54.5- 83.9)	2.09	0.55
> 153.0246	59.7 (47.5- 71.1)	70.7 (54.5- 83.9)	2.04	0.57
> 155.4	59.7 (47.5- 71.1)	73.2 (57.1- 85.8)	2.23	0.55
> 158.2157	58.3 (46.1- 69.8)	73.2 (57.1- 85.8)	2.17	0.57
> 158.9078	56.9 (44.7- 68.6)	73.2 (57.1- 85.8)	2.12	0.59
> 163.0607	55.6 (43.4- 67.3)	73.2 (57.1- 85.8)	2.07	0.61
> 163.176	55.6 (43.4- 67.3)	75.6 (59.7- 87.6)	2.28	0.59
> 170.3282	55.6 (43.4- 67.3)	78.0 (62.4- 89.4)	2.53	0.57
> 171.2511	54.2 (42.0- 66.0)	78.0 (62.4- 89.4)	2.47	0.59
> 173.2	54.2 (42.0- 66.0)	80.5 (65.1- 91.2)	2.78	0.57
> 173.9043	52.8 (40.7- 64.7)	80.5 (65.1- 91.2)	2.7	0.59
> 174.6	51.4 (39.3- 63.3)	80.5 (65.1- 91.2)	2.63	0.6
> 175.8	50.0 (38.0- 62.0)	80.5 (65.1- 91.2)	2.56	0.62
> 186.8244	50.0 (38.0- 62.0)	82.9 (67.9- 92.8)	2.93	0.6
> 186.9397	50.0 (38.0- 62.0)	85.4 (70.8- 94.4)	3.42	0.59
> 188.324	48.6 (36.7- 60.7)	85.4 (70.8- 94.4)	3.32	0.6

> 189.3622	47.2 (35.3- 59.3)	85.4 (70.8- 94.4)	3.23	0.62
> 193.1	45.8 (34.0- 58.0)	85.4 (70.8- 94.4)	3.13	0.63
> 193.169	45.8 (34.0- 58.0)	87.8 (73.8- 95.9)	3.76	0.62
> 194.2073*	45.8 (34.0- 58.0)	90.2 (76.9- 97.2)	4.7	0.6
> 202.6284	44.4 (32.7- 56.6)	90.2 (76.9- 97.2)	4.56	0.62
> 205.2816	43.1 (31.4- 55.3)	90.2 (76.9- 97.2)	4.41	0.63
> 205.5	40.3 (28.9- 52.5)	90.2 (76.9- 97.2)	4.13	0.66
> 217.8556	38.9 (27.6- 51.1)	90.2 (76.9- 97.2)	3.99	0.68
> 222.6	37.5 (26.4- 49.7)	90.2 (76.9- 97.2)	3.84	0.69
> 223.162	36.1 (25.1- 48.3)	90.2 (76.9- 97.2)	3.7	0.71
> 225.8	34.7 (23.9- 46.9)	90.2 (76.9- 97.2)	3.56	0.72
> 226	33.3 (22.7- 45.4)	90.2 (76.9- 97.2)	3.42	0.74
> 230.4	31.9 (21.4- 44.0)	90.2 (76.9- 97.2)	3.27	0.75
> 233.7749	30.6 (20.2- 42.5)	90.2 (76.9- 97.2)	3.13	0.77
> 249.5789	29.2 (19.0- 41.1)	90.2 (76.9- 97.2)	2.99	0.78
> 251.6554	29.2 (19.0- 41.1)	92.7 (80.1- 98.4)	3.99	0.76
> 255.7	29.2 (19.0- 41.1)	95.1 (83.4- 99.3)	5.98	0.74
> 261.5761	27.8 (17.9- 39.6)	95.1 (83.4- 99.3)	5.69	0.76
> 264.114	26.4 (16.7- 38.1)	95.1 (83.4- 99.3)	5.41	0.77
> 264.3447	25.0 (15.5-36.6)	95.1 (83.4- 99.3)	5.12	0.79

> 272.6505	23.6 (14.4-35.1)	95.1 (83.4- 99.3)	4.84	0.8
> 277.6109	22.2 (13.3-33.6)	95.1 (83.4- 99.3)	4.56	0.82
> 280.0334	20.8 (12.2-32.0)	95.1 (83.4- 99.3)	4.27	0.83
> 281	19.4 (11.1-30.5)	95.1 (83.4- 99.3)	3.99	0.85
> 281.8791	18.1 (10.0-28.9)	95.1 (83.4- 99.3)	3.7	0.86
> 282.3405	16.7 (8.9-27.3)	95.1 (83.4- 99.3)	3.42	0.88
> 290.4	15.3 (7.9-25.7)	95.1 (83.4- 99.3)	3.13	0.89
> 296.9	13.9 (6.9-24.1)	95.1 (83.4- 99.3)	2.85	0.91
> 315.7942	12.5 (5.9-22.4)	95.1 (83.4- 99.3)	2.56	0.92
> 322.9464	11.1 (4.9-20.7)	95.1 (83.4- 99.3)	2.28	0.93
> 326.6379	9.7 (4.0-19.0)	95.1 (83.4- 99.3)	1.99	0.95
> 336.5586	9.7 (4.0-19.0)	97.6 (87.1- 99.6)	3.99	0.93
> 342.7879	8.3 (3.1-17.3)	97.6 (87.1- 99.6)	3.42	0.94
> 347.1715	8.3 (3.1-17.3)	100.0 (100.0-100.0)		0.92
> 349.8248	6.9 (2.3-15.5)	100.0 (100.0-100.0)		0.93
> 372.2041	5.6 (1.6-13.6)	100.0 (100.0-100.0)		0.94
> 424.5765	4.2 (0.9-11.7)	100.0 (100.0-100.0)		0.96
> 464.3749	2.8 (0.4-9.7)	100.0 (100.0-100.0)		0.97
> 469.9121	1.4 (0.2-7.5)	100.0 (100.0-100.0)		0.99
> 504.6347	0.0 (0.0-0.0)	100.0 (100.0-100.0)		1

CI=confidence intervals; LR+ = Likelihood ratio for a positive test result; LR- = Likelihood ratio for a negative test result.

Appendix 7.

Autoinflammatory Diseases (AID) genes contained on the "Vasculitis and Inflammation Panel" (VIP).

Gene symbol	Gene name	Transcript
ACP5	Acid phosphatase-5/tartrate-resistant phosphatase	NM_001111034
ACTA2	Actin alpha 2	NM_001613
ADA2	Cat eye syndrome chromosome region 1/Adenosine deaminase 2	NM_001282225
ADAM17	ADAM Metallopeptidase Domain 17	NM_003183
ADAR	Adenosine deaminase acting on RNA	NM_001111
AICDA	Activation-induced cytidine deaminase	NM_020661
AIRE	Autoimmune Regulator	NM_000383
AP1S3	Adaptor Related Protein Complex 1 Sigma 3 Subunit	NM_001039569
AP3B1	Adaptor Related Protein Complex 3 Beta 1 Subunit	NM_003664
APOA1-AS	Apolipoprotein A1	NM_000039
APOA2	Apolipoprotein A2	NM_001643
APOA4	Apolipoprotein A4	NM_000482

APOC2	Apolipoprotein C2	NM_000483
APOC3	Apolipoprotein C3	NM_000040
APOE	Apolipoprotein E	NM_000041
APP	Amyloid Beta Precursor Protein	NM_000484
B2M	Beta-2-Microglobulin	NM_004048
BLOC1S6	Biogenesis Of Lysosomal Organelles Complex 1 Subunit 6 (BLOC1S6)	NM_012388
BMPR2	Bone morphogenetic protein type II receptor	NM_001204
BTK	Bruton's tyrosine kinase	NM_000061
C1QA	Complement C1q A Chain	NM_015991
C1QB	Complement C1q B Chain	NM_000491
C1QC	Complement C1q C Chain	NM_172369
C1R	Complement C1r	NM_001733
C2	Complement C2	NM_000063
C3	Complement C3	NM_000064
C5	Complement C5	NM_001735
C6	Complement C6	NM_000065
C7	Complement C7	NM_000587
C8A	Complement C8 Alpha Chain	NM_000562
C8B	Complement C8 Beta Chain	NM_000066
C9	Complement C9	NM_001737

CARD14	Caspase Recruitment Domain Family Member 14	NM_024110
CASP10	Caspase 10	NM_032977
CASP8	Caspase 8	NM_033355
CBL	Cbl Proto-Oncogene, E3 Ubiquitin Protein Ligase	NM_005188
CBS	Cystathionine beta synthase	NM_000071
CD40LG	CD40 antigen ligand	NM_000074
CD70	Tumor Necrosis Factor Ligand Superfamily Member 7	NM_001252
CFH	Complement factor H	NM_000186
CFHR5	Complement factor H-related protein 5	NM_030787
CFI	Complement factor 1	NM_000204
CFP	Complement Factor Properdin	NM_002621
COL3A1	Collagen Type III Alpha 1 Chain	NM_000090
COL4A1	Collagen Type IV Alpha 1 Chain	NM_001845
COL5A1	Collagen Type V Alpha 1 Chain	NM_000093
COL5A2	Collagen Type V Alpha 2 Chain	NM_000393
COL7A1	Collagen Type VII Alpha 1 Chain	NM_000094
COPA	Coatomer subunit alpha	NM_001098398 NM_004371
CORO1A	Coronin, actin binding protein, 1A	NM_007074
CPT2	Carnitine palmitoyltransferase 2	NM_000098

CST3	Cystatin C3	NM_000099
CTC1	CTS telomere maintenance complex component 1	NM_025099
CTPS1	Cytidine 5’ triphosphate synthase 1	NM_001905
CYBA	Cytochrome b alpha chain	NM_000101
CYBB	Cytochrome b beta chainp91-phox	NM_000397
DCLRE1C	DNA cross-link repair 1c	NM_001033855
DNASE1	Deoxyribonuclease 1	NM_005223
DNASE1L3	Deoxyribonuclease I-like 3	NM_004944
DNASE2	deoxyribonuclease II, lysosomal	NM_001375
DOCK8	Dedicator of cytokinesis 8	NM_203447
EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2; also referred to as Fibulin 4; FBLN4	NM_016938
ELANE	Elastase, neutrophil-expressed	NM_001972
ELN	Elastin	NM_001278939
FAS	Tumour necrosis factor receptor superfamily member 6	NM_000043
FASLG	Tumor necrosis factor ligand superfamily member 6 (FAS ligand)	NM_000639
FBN1	Fibrillin 1	NM_000138
FBN2	Fibrillin 2	NM_001999
FERMT1	Ferritin family member 1	NM_017671
FGA	Fibrinogen Alpha Chain	NM_000508

FLNA	Filamin A	NM_001456
FOXE3	Forkhead Box E3	NM_012186
FOXP3	Forkhead box P3	NM_014009
G6PC3	Glucose-6-phosphatase 3	NM_138387
GATA2	GATA-binding protein 2	NM_032638 NM_001145661
GLA	Alpha-galactosidase A	NM_000169
GSN	Gelsolin	NM_001127662
GUCY1A3	Guanylate Cyclase 1 Soluble Subunit Alpha	NM_000856
GUCY2C	Guanylate cyclase 2C	NM_004963
HFE	Hemochromatosis	NM_000410
HPS1	Hermansky-Pudlak syndrome type 1	NM_000195
HPS4	Hermansky-Pudlak syndrome type 4	NM_022081
HPS6	Hermansky-Pudlak syndrome type 6	NM_024747
HTR1A	5-Hydroxytryptamine Receptor 1A	NM_000524
HTRA1	HtrA serine peptidase-1 gene	NM_002775
ICOS	Inducible T-cell co-stimulator	NM_012092
IFIH1	Interferon induced helicase C domain containing protein 1	NM_022168
IFNGR1	Interferon gamma receptor 1	NM_000416
IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)	NM_005534

IKBKG	IKK-gamma	NM_003639
IL10	Interleukin 10	NM_000572
IL10RA	Interleukin 10 receptor, alpha	NM_001558
IL10RB	Interleukin 10 receptor, beta	NM_000628
IL1RN	Interleukin 1 receptor antagonist	NM_173842
IL2RA	Interleukin 2 receptor, alpha chain	NM_000417
IL31RA	Interleukin 31 Receptor A	NM_139017
IL36RN	Interleukin 36 receptor antagonist	NM_173170
IRF8	Interferon Regulatory Factor 8	NM_002163
ISG15	ISG15 Ubiquitin-Like Modifier	NM_005101
ITGB2	Beta-2 integrin chain	NM_000211
LACC1	Laccase domain containing 1	NM_001128303
LMNA	Laminin A	NM_170707
LOX	Lysyl Oxidase	NM_001178102 NM_001317073 NM_002317
LPIN2	Lipin 2	NM_014646
LRBA	Lipopolysaccharide-responsive and beige-like anchor brotein	NM_001199282
LYN	Tyrosine-Protein Kinase	NM_002350
LYST	Lysosomal trafficking regulator	NM_000081

LYZ	Lysozyme	NM_000239
MAGT1	Magnesium Transporter 1	NM_032121
MASP2	Mannose-binding lectin serine protease 2	NM_006610
MAT2A	Methionine Adenosyltransferase 2A	NM_005911
MBL2	Mannose-binding lectin	NM_000242
MEFV	MEditerranean FeVer	NM_000243
MFAP5	Microfibrillar Associated Protein 5	NM_003480
MVK	Mevalonate Kinase	NM_000431
MYD88	Myeloid Differentiation Primary Response 88	NM_001172569 NM_002468
MYH11	Myosin, Heavy Chain 11, Smooth Muscle	NM_001040113
MYLK	Myosin Light Chain Kinase	NM_053025
NCF2	Neutrophil cytosol factor 2	NM_000433
NCF4	Neutrophil cytosol factor 4	NM_000631
NF1	Neurofibromin 1	NM_000267
NLRC4	NLR Family CARD Domain Containing 4	NM_021209
NLRP1	NLR Family Pyrin Domain Containing 1	NM_033004
NLRP12	NLR Family Pyrin Domain Containing 12	NM_144687
NLRP3	NLR Family Pyrin Domain Containing 3	NM_001243133
NLRP6	NLR Family Pyrin Domain Containing 6	NM_138329

NLRP7	NLR Family Pyrin Domain Containing 7	NM_001127255
NOD2	Nucleotide-binding oligomerization domain 2	NM_022162
NOTCH1	Notch 1	NM_017617
NOTCH3	Notch 3	NM_000435
NRAS	Neuroblastoma ras	NM_002524
OSMR	Oncostatin M Receptor	NM_001323505 NM_001323506 NM_003999
OTULIN	OUT deubiquitinase with linear linkage specificity	NM_138348
PIK3CD	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta	NM_005026
PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha	NM_181504
PLCG2	Phospholipase C, Gamma-2	NM_002661
PLOD1	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	NM_001316320
POMP	Proteasome maturation protein	NM_015932
PRF1	Perforin	NM_005041
PRG4	Proteoglycan 4	NM_005807
PRKCD	Protein Kinase C, Delta	NM_006254
PRKG1	Protein kinase, cGMP-dependent, type I	NM_001098512
PSMA3	Proteasome Subunit Alpha 3	NM_002788

PSMB4	Proteasome Subunit Beta 4	NM_002796
PSMB8	Proteasome Subunit Beta 8	NM_148919
PSMB9	Proteasome Subunit Beta 9	NM_002800
PSTPIP1	Proline-Serine-Threonine Phosphatase Interacting Protein 1	NM_003978
PTEN	Phosphatase and tensin homolog	NM_000314 NM_001304717
PYCARD	PYD and CARD domain containing	NM_013258
RAB27A	RAB27A, Member RAS Oncogene Family	NM_004580
RAG1	Recombinant activating gene 1	NM_000448
RANBP2	RAN Binding Protein 2	NM_006267
RASGRP1	RAS Guanyl Releasing Protein 1	NM_001128602 NM_005739
RBCK1	RANBP2-Type And C3HC4-Type Zinc Finger Containing 1	NM_031229
RET	Ret Proto-Oncogene	NM_020975
RHOD	Ras Homolog Family Member D	NM_014578
RNASEH2A	Ribonuclease H2 subunit A	NM_006397
RNASEH2B	Ribonuclease H2 subunit B	NM_024570
RNASEH2C	Ribonuclease H2 subunit C	NM_032193
RNF213	Ring Finger Protein 213	NM_001256071
SAMHD1	SAM-domain and HD-containing protein 1	NM_015474

SCN9A	Sodium channel, voltage-gated, type IX, alpha subunit	NM_002977
SERPING1	Serpin Peptidase Inhibitor, Clade G (C1 Inhibitor), Member 1	NM_000062
SH2D1A	SH2-domain protein 1a (Slam-associated protein)	NM_002351
SH3BP2	SH3-domain binding protein 2	NM_003023
SKI	v-ski avian sarcoma viral oncogene homolog	NM_003036
SKIV2L	Superkiller viralicidic activity 2-like	NM_006929
SLC29A3	Solute carrier family 29 (nucleoside transporter), member 3	NM_018344
SLC2A10	Solute carrier family 2 (facilitated glucose transporter), member 10	NM_030777
SLC37A4	Solute carrier family 37 (glucose-6-phosphate transporter), member 4	NM_001467
SLC7A7	Solute Carrier Family 7 Member 7	NM_001126106
SMAD2	SMAD Family Member 2	NM_005901
SMAD3	SMAD family member 3	NM_005902
SMAD4	SMAD family member 4	NM_005359
STK4	Serine/Threonine Kinase 4	NM_006282
STX11	Syntaxin 11	NM_003764
STXBP2	Syntaxin binding protein 2	NM_006949
TGFB2	transforming growth factor, beta 2	NM_001135599
TGFB3	Transforming growth factor, beta-3	NM_003239
TGFBI	Transforming Growth Factor-Beta Induced	NM_000358

TGFBR1	Transforming growth factor-beta receptor, type 1	NM_004612
TGFBR2	Transforming growth factor-beta receptor, type 2	NM_001024847
TMEM107	Small Nucleolar RNA, C/D Box 118	NR_033294
TMEM173	Transmembrane protein 173	NM_198282
TNFAIP3	TNF Alpha Induced Protein 3	NM_001270507
TNFRSF11A	TNF Receptor Superfamily Member 11a	NM_003839
TNFRSF1A	TNF Receptor Superfamily Member 1A	NM_001065
TRAP1	TNF Receptor Associated Protein 1	NM_016292
TREX1	Three prime repair exonuclease 1	NM_016381
TRIM28	Tripartite Motif Containing 28	NM_005762
TRNT1	tRNA nucleotidyl transferase, CCA-adding, 1	NM_182916
TTC37	Tetratricopeptide repeat domain 37	NM_014639
TTR	Transthyretin	NM_000371
UNC13D	Unc-13 Homolog D	NM_199242
USB1	U6 SnRNA Biogenesis Phosphodiesterase 1	NM_024598
USP18	Ubiquitin-specific protease 18	NM_017414
VPS13B	Vacuolar protein sorting 13 homolog B (yeast)	NM_017890
WAS	Wiskott-Aldrich syndrome	NM_000377
WDR1	WD Repeat containing 1/Actin-interacting protein 1	NM_005112
XIAP	X-linked inhibitor of apoptosis	NM_001167

YY1AP1	YY1 Associated Protein 1	NM_001198906 NM_139118

Appendix 8

Dose escalation schedule for patients with eGFR ≥120 mL/min/1.73 m^2

Initial Dose

Weight Class[a]	eGFR (mL/min/1.73 m^2)	Morning Dose	Afternoon Dose	Evening Dose	Total Daily Dose[b]	Duration[c]	Min/Max Dose (mg/kg)[d]	Dosing Frequency
<20 kg	≥120	2 mg	2 mg	2 mg	6 mg	72 hours	0.3/NA	TID
20-40 kg	≥120	3 mg	—	3 mg	6 mg	72 hours	0.15/0.3	BID
>40 kg	≥120	4 mg	—	4 mg	8 mg	72 hours	NA/0.2	BID

First Dose Escalation

Weight Class[a]	eGFR (mL/min/1.73 m^2)	Morning Dose	Afternoon Dose	Evening Dose	Total Daily Dose[b]	Duration[c]	Min/Max Dose (mg/kg)[d]	Dosing Frequency
<20 kg	≥120	2 mg	2 mg	2 mg	8 mg	72 hours	0.4/ NA	QID
20-40 kg	≥120	3 mg	2 mg	3 mg	8 mg	72 hours	0.2/0.4	TID
>40 kg	≥120	5 mg	—	5 mg	10 mg	72 hours	NA/ 0.25	BID

Second Dose Escalation (only in patients > 40 kg)

Weight Class[a]	eGFR (mL/min/1.73 m^2)	Morning Dose	Afternoon Dose	Evening Dose	Total Daily Dose[b]	Duration[c]	Min/Max Dose (mg/kg)[d]	Dosing Frequency
>40 kg	≥120	6 mg	—	6 mg	12 mg	72 hours	NA/0.3	BID

Abbreviations: max = maximum; min = minimum; NA = not applicable.
— indicates no further dose escalation allowed.

10 Publications from this thesis

Papadopoulou C., Eleftheriou D. "Janus Kinase 1/2 inhibition with baricitinib in the treatment of Juvenile dermatomyositis".Brain. 2019 Feb 1

Papadopoulou C., McCann LJ. "The vasculopathy of Juvenile Dermatomyositis". Front Pediatr. 2018 Oct 9

Papadopoulou C., Wedderburn L. "Treatment of Juvenile Dermatomyositis: An Update". Pediatric Drugs. 2017 May 26

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