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Elucidating the Mechanisms of Thromboembolism and Structural Arterial Disease in Children with Inflammatory Bowel Disease

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I, James Bonner, 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.

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Thesis Abstract

Inflammatory bowel disease (IBD) is a group of life-long relapsing and remitting inflammations of the gastrointestinal (GI) tract, with complex aetiology and no known cure. Incidence world-wide is increasing, especially among paediatric-onset cases. In addition to known primary morbidities, recent epidemiological evidence suggests an increased life-time risk of cardiovascular events in IBD patients, however the mechanism for this is unknown. This thesis attempts to investigate the hypothesis that chronic inflammation in IBD promotes vascular dysfunction, which leads to increased cardiovascular risk, and is detectable in paediatric IBD. Mechanisms of endothelial microparticle (MP) formation were investigated, and it was found cytokines associated with IBD stimulate MP release in a synergistic manner. Additionally, markers of endothelial injury, hypercoagulability and circulating microparticles (MPs) were assessed in paediatric IBD patients and compared to healthy controls. Circulating endothelial cells (CECs) were raised in paediatric IBD, with even inactive patients having significantly higher CECs. This was corroborated by increased circulating levels of MPs, particularly those derived from neutrophils, the endothelium and those expressing tissue factor (TF). Paradoxically, plasma from patients showed delayed thrombin generation, as well as MP-mediated thrombin generation, measured by an *in vitro* assay. Patients also showed increased plasma activity of TF and derangement of the TF/TFPI axis. Patients were assessed for structural arterial disease by assessment of carotid-femoral pulse wave velocity (PWV), but showed no difference to healthy controls. Finally, assessment of plasma cytokines and vascular injury markers by multi-plex electrochemiluminescent assay showed an increase in multiple pro-inflammatory cytokines in paediatric IBD. In summary, these novel findings suggest an increase in vascular injury in paediatric IBD along with promotion of a pro-thrombotic state. These findings provide a unique insight into the cardiovascular complications of IBD patients.

Abbreviations

Abbreviation	Definition
5-ASA	5-aminosalicylic acid
6-MP	6-mercaptopurine
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	Analysis of Variance
AnV	Annexin V
APC	Allophycocyanin
ATP	Adenosine Triphophate
BMI	Body Mass Index
BP	Blood Pressure
BSA	Bovine Serum Albumin
BV	Brilliant Violet
CD	Crohn's Disease
CD11	Cluster of Differentiation (example)
CEC	Circulating Endothelial Cells
CRP	C-reactive Protein
CSK	C-terminal Src Kinase
CV	Coefficient of Variation
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EEN	Exclusive Enteral Nutrition
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked Immunosorbant Assay
ERM	Ezrin, Radixin, Moesin
ESR	Eryhtrocyte Sedimentation Rate
ETP	Endogenous Thrombin Potential
FACS	Flow-assissted Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FMD	Flow-mediated Dilation
FMO	Fluorescence Minus One

FSc	Forward Scatter
GAP	GTPase Activating Protein
GDP	Guanine Diphosphate
GEF	Guanine nucleotide Exchange Factor
GI	Gastrointestinal
GOSH	Great Ormond Street Hospital
GPCR	G-protein Coupled Receptor
GSK	GSK269962
GTP	Guanine Triphosphate
GWAS	Genome-wide Association Study
HDL	High Density Lipoprotein
HUVEC	Human Umbilical Vein Endothelial Cell
IBD	Inflammatory Bowel Disease
IBD-U	Inflammatory Bowel Disease Unclassified
IC	Indeterminate Colitis
ICAM-1	Intercellular Adhesion Molecule-1
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
IL	Interleukin
LDL	Low Density Lipoprotein
LIMK2	LIM domain Kinase 2
LPS	Lipopolysaccharide
MAP	Mitogen-activated Protein
miRNA	Micro Ribonucleic Acid
mmHg	Millimetres of Mercury
MMP	Matrix Metalloprotease
MP	Microparticle
MPFP	Microparticle-free Plasma
NFκB	Nuclear Factor kappa B
NMP	Neutrophil Microparticle
PBS	Phosphate-buffered Saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PerCP	Peridinin Chlorophyll Protein Complex

pH	phenol Red
PMA	Phorbol 12-myristate 13-acetate
PPP	Platelet-poor Plasma
PS	Phosphatidylserine
PWV	Pulse Wave Velocity
RA	Rheumatoid Arthritis
Rho-GDI	Rho-GDP Disassociation Inhibitor
ROCK-1	Rho-associated, coiled coil containing protein kinase
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SAA	Serum Amyloid A
SSc	Side Scatter
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TGF-β	Transforming Growth Factor Beta
TNF-α	Tumour Necrosis Factor Alpha
TPMT	Thiopurine Methyltransferase
TRPC1	Transient Receptor Potential Channel 1
UC	Ulcerative Colitis
UCH	Univeristy College Hospital
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein

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1. Introduction

1.1 Inflammatory Bowel Disease (IBD)

Inflammatory Bowel Disease (IBD) is a group of chronic life-long relapsing and remitting inflammatory diseases of the gastrointestinal (GI) tract, with no current known cure. These are categorised most commonly as Crohn's Disease (CD), Ulcerative Colitis (UC), or IBD unclassified (IBD-U), with multiple potential causes, risk factors and genetic predispositions (Abegunde et al., 2016). The cause is thought to be a combination of genetic predisposition, immune dysregulation, impaired intestinal barrier function and dysbiosis of the mucosal microbiota. General symptoms consist of diarrhoea, abdominal pain, loss of energy and weight and bleeding as a result of mucosal and systemic inflammation, though symptoms can be varied between patients and over time within the same patient. There is currently no biomarker available for the differentiation of IBD into its subtypes, and classification is achieved predominantly through the location and histological appearance of inflammation.

1.1.1 Crohn's Disease (CD)

CD was first described by, and named after, Burrill Bernard Crohn and two colleagues in 1932, describing "regional ileitis" found in patients at Mount Sinai Hospital in New York (Crohn et al., 2000). Common symptoms of CD are abdominal pain, fever, diarrhoea, anal fistulae, and anal tenesmus (feeling of incomplete defection). Endoscopically, the disease is characterised by alternating patches of inflamed and uninflamed tissue in affected areas of the GI tract, called "skip lesions" (Patman, 2014), CD is classified as ileal, colonic, or ileocolonic if both sections of the bowel are inflamed. Unlike UC, CD can involve the entire GI tract with orofacial granulomatosis a potential sign of subclinical CD (Rowland et al., 2010).

With more severe manifestations of CD, strictures, or narrowing of the intestines can develop, due to wall thickening caused by inflammation. These can develop to an extent where

blockage of the GI tract occurs. In such conditions, surgical resection is often the only choice of treatment (Mao et al., 2014).

1.1.2   Ulcerative Colitis (UC)

UC was the first of the IBDs to be described, first appearing in medical literature by name in 1859 by Sir Samuel Wilks (De Dombal, 1968). All patients were originally thought to have GI tuberculosis, though it soon became clear that this colitis was a separate entity. Common symptoms of UC include abdominal pain and diarrhoea, rectal bleeding, anaemia, and anal tenesmus. Unlike CD, UC tends to manifest as continuous inflammation in the GI tract, and is restricted to the large intestine (bowel), varying from distal colitis to pancolitis with involvement of the whole bowel. Due to lack of involvement of the small intestine, patients with UC are less likely to suffer weight loss than with CD. Complications of severe or late-stage UC include bowel perforation and toxic megacolon, potentially requiring a total colectomy (Bohl and Sobba, 2015).

1.1.3   IBD-Unclassified (IBD-U)/Indeterminate Colitis (IC)

While the majority of IBD patients fit a stereotypical CD or UC-like phenotype, when it is difficult to classify a patient into one category or the other by endoscopic, histological and radiologic findings, they are classed as IBD-unclassified (IBD-U) or indeterminate colitis (IC) (Guindi and Riddell, 2004). The use of the term IC originally had a precise definition, of bowel inflammation post-colectomy, but now generally refers to IBD that cannot be classified as CD or UC (Tremaine, 2007). This vague diagnosis means that descriptors are added, determining the location of inflammation and whether it is "Crohns-like" or "UC-like". In adults, it is suggested that the majority of cases of IC are in fact UC, with either unusual presentation or insufficient pathological information to make a definite UC diagnosis (Odze, 2015).

An important feature of IC is that a paediatric IBD patient is twice as likely to have an IC diagnosis than an adult (12.7% vs 6.0%, Prenzel and Uhlig, 2009). It is unclear however if this is due to IC being associated with paediatric onset, or if it is simply due to lack of diagnostic

information allowing a more precise diagnosis. As the disease progresses and more information becomes available, some IBD-U/IC diagnoses are re-classififed to either UC or CD. A small retrospective study found half of the paediatric IC patients followed up for 5 years were re-classified, with the majority becoming UC (Newby et al., 2008).

1.1.4 Extraintestinal manifestations of IBD

While IBD is predominantly a disease of the intestinal tract, there are features and symptoms that can occur elsewhere in the body. The most common areas of extraintestinal manifestations are the joints, skin and eyes, but can also affect the hepatobiliary system, lungs and heart (Ott and Schölmerich, 2013). Some are related to IBD, correlate with disease activity and subside with the treatment of IBD (e.g. peripheral arthritis or erythema nodosum). Others are independent of IBD disease activity and require treatments directed to them specifically, such as primary sclerosing cholangitis and ankylosing spondylitis. A population based study of the most common extraintestinal manifestations of IBD show 6.2% of the population studied had at least one (Bernstein et al., 2001), though with extraintestinal manifestations potentially correlating with IBD disease activity and the relapsing and remitting nature of the disease, it is presumed that actual prevalence of extrainestinal manifestations would be highly variable. The overall pathogenesis of these features is thought to be inflammation within the intestine causing proximal inflammation, either immune cell-mediated or cytokine-mediated (Ott and Schölmerich, 2013).

1.2 Incidence/Prevalence of IBD

Current mean prevalence of IBD in western countries is 1-5 in 1000 (Gasparetto and Guariso, 2013), though a steady increase in incidence (for Europe, 24.3 per 100,000 person-years in UC, 12.7 per 100,000 person-years in CD), especially amongst paediatric populations has been noted in the last four decades (Lovasz et al., 2014; Chuang et al., 2013; (Martín-de-Carpi et al., 2014); Molodecky et al., 2012). A systematic review has shown that while incidence of paediatric IBD varies around the world, there is a definite trend to an increase, with the

majority of extra cases being CD (Benchimol et al., 2011). Part of this increase could be accounted for by increased knowledge of the disease and diagnosis, but the rate of increase suggests there are other factors at play.

An interesting variation in IBD incidence is found when looking at latitude, with UC and CD in the USA (Khalili et al., 2012) and Europe (Shivananda et al., 1996) showing increased incidence with increased latitude. One hypothesis offered for these findings is the potential impact of reduced sun exposure, and therefore vitamin D status, with a recent meta-analysis showing a significant association between IBD and vitamin D deficiency (Del Pinto et al., 2015). However, this association does not show a causative link, and latitude did not influence the relationship between IBD and vitamin D status. Confirmation of this hypothesis would require either a randomised controlled trial (RCT), or a more detailed longitudinal study.

A further observation on IBD incidence is the variation seen in prevalence across socio-economic status. A 34 centre cohort across east and west Europe showed those in western Europe were twice as likely to have UC and CD as those in eastern Europe (Burisch et al., 2014). This combined with the observation of the disease emerging in developing economies (Ng et al., 2013) demonstrates an environmental component to developing IBD, but also points to the possibility of "Western lifestyle" being a risk factor. Within this, many features including a sedentary lifestyle, dietary changes, increased exposure to pollutants and increased exposure to antibiotics may contribute to driving these increases, particularly amongst the paediatric population.

1.3 IBD in children

While the majority of IBD is diagnosed between the ages of 20 and 40, the proportion that are diagnosed as children is increasing (Malmborg and Hildebrand, 2016). While environmental and genetic components are thought to contribute to IBD pathogenesis, the younger the age of onset, the larger the contribution of genetics is likely to be. The majority of paediatric IBD patients are diagnosed between the ages of 11 and 16, but those diagnosed between the ages

of 5 and 11 show increased disease severity, extent, and burden than children diagnosed later (Gasparetto et al., 2016). This demonstrates that age of onset can influence the course of the disease, and lends further evidence to the increased role of genetics in paediatric IBD. Similarly to adult IBD, there is a large discrepancy between the clinical symptoms and biomarkers of inflammation in paediatric IBD, with GI inflammation continuing even in the absence of symptoms (Hoekman et al., 2016).

Due to the life-long nature of IBD, paediatric patients face a unique challenge. As they become older with the disease, becoming adolescents and then young adults, they have to transition from paediatric care to adult care. How a patient transitions is very important in maintaining health, and minimising disruption to growth, education and personal development (Goodhand et al., 2011). Surveying adult and paediatric gastroenterologists identified concerns with the current state of transition care (Wright et al., 2014). Only 23% of respondents felt adolescent patients were adequately prepared to transition to adult care, with poor medical handover and lack of responsibility for their own care being cited as major reasons for lack for preparedness.

1.4 Genetics

While there is no known overall cause for most cases of IBD, several major genome-wide association studies (GWAS) have identified genetic loci associated with the disease (de Lange and Barrett, 2015). Currently 163 loci are known that significantly correlate with IBD (Jostins et al., 2012), with ~75% of them shared with other immune-mediated diseases, such as rheumatoid arthritis (RA) (Parkes et al., 2013).

Initial studies in twins showed a coefficient of heritability of 0.75 for CD and 0.67 for UC, though GWAS have shown a far lower heritability (Gordon et al., 2015). The main hypothesis for this discrepancy is the failure of twin studies to take into account the shared environment of twins, and therefore over-estimate the influence of genetics. Some of the polymorphisms that are associated with earlier diagnosis of CD have been shown in the nucleotide-binding

oligomerization domain-containing protein 2 (NOD2) gene, POU5F1 (an embryonic stem cell differentiation factor), and tumour necrosis factor super family 15 (TNFSF15) (Connelly et al., 2015). While there is a large overlap between CD and UC in terms of symptoms and potential causes, there are unique elements to the mechanisms of pathogenesis for both. However, none point to a single cause, with intestinal barrier function, adaptive and innate immunity being implicated in both conditions.

While in the vast majority of cases there is no single genetic locus associated with IBD, the information generated by GWAS can still make a difference to clinical practice. For example, information gleaned from GWAS could in theory allow genetic screening for risk factors, though with small individual effect sizes of loci, the predictive power of this would be low. The pathways implicated by GWAS however could influence the use of novel drugs and development of new ones (Parkes et al., 2013). This is the case with ustekinumab, an anti-IL-12/23 monoclonal antibody recently shown to be effective in the treatment of IBD (Sandborn et al., 2012). The IL-12/23 axis had been previously implicated by GWAS as the IL-23 receptor was an associated gene (Duerr et al., 2006), which directed interest to the therapeutic potential of this pathway.

While GWAS have identified many associated loci with IBD, there are however very rare cases, particularly in the young, where a monogenic cause can be found.

1.4.1   Monogenic causes of IBD

In the rare case of a monogenic cause of IBD, the age of onset tends to be very young, typically <6 years (Capriati et al., 2016). There is debate as to whether these diseases are IBD, or monogenic intestinal inflammation with IBD-like symptoms, as the vast majority of IBD cases do not have a single cause. However, the genes involved in these diseases can help with the understanding of IBD (Uhlig, 2013). Similarly to that found in GWAS, monogenic causes of IBD include pathways involved in intestinal barrier function, leukocyte maturation, and regulatory T-cell function. The main difference with those found in GWAS is the mutations found are

deleterious and completely disrupt the function of the gene in question. Due to the very early age of onset, and specificity of disease in monogenic cases, they will not be discussed further or included in any studies investigating paediatric IBD.

1.5 IBD and the Microbiome

The microbiota in the intestinal lumen is hugely complex, containing more bacterial cells than make up the whole human body. The typical intestinal microbiota contains 10^{14} bacteria, weighs 1.5kg, and contains over 1000 different species (Sommer and Bäckhed, 2013). Advances in 16S ribosomal RNA (rRNA), and then whole genome, sequencing technologies have enabled the investigation of the distribution of species in faecal samples, the majority of which cannot be cultured in the laboratory to be investigated (Ranjan et al., 2016).

Investigations into the diversity of the microbiome have shown that generally, the more diverse the species present, the more robust the community is to change and the healthier the individual is. This is seemingly in turn influenced by the diversity of the diet (Heiman and Greenway, 2016). Better health may also be related to the diverse commensal microbiota out-competing potential pathogens for resources, leaving them no space to colonise. Cammarota *et al* comprehensively summarise the involvement of the gut microbiota on IBD pathogenesis, highlighting that the interaction between the host immune system and bacteria in the gut has a large influence over their potential pathogenicity. This is additionally highlighted in the fact that IBD patients tend to have far lower diversity of bacteria in their microbiome than healthy people (Prideaux et al., 2013). CD is most strongly associated with reduced bacterial diversity, with UC also showing reduced diversity in the Chinese population. Whether this is a cause or effect of IBD has yet to be determined, but has led to the investigation of faecal transplants in the treatment of IBD. A faecal transplant involves the removal of the contents of the intestines in the patient by laxative, and introduction of the faeces of a healthy person. A systematic review and meta-analysis of faecal transplant to treat IBD found it to be safe, but with variable efficacy (Colman and Rubin, 2014). This is unsurprising as the variable nature of the disease

and microbiota of different patients mean the potential effect of a pathogenic microbiota on IBD pathogenesis is also highly variable. While this treatment may help some with IBD, standardisation of the procedure and the difficult definition of what a healthy donor microbiota is mean its use is still rare.

1.6 Treatments

Until recently the goal of treatment of IBD was to eliminate symptoms and maintain remission. Remission was defined as the absence of symptoms. However, it has been shown that symptoms and histological inflammation do not necessarily correlate, and ongoing histological inflammation can lead to disease progression in CD (Cellier et al., 1994) and UC (Gupta et al., 2007), even in the absence of symptoms. The concept of "deep remission" or mucosal healing as a treatment goal (Zallot and Peyrin-Biroulet, 2013) has led to more aggressive treatment of IBD, with improved outcomes in corticosteroid use, time spent in remission, and rates of colectomy and bowel resection (Walsh et al., 2014). Combined with new treatment targets, therapeutics for IBD have expanded over the past 20 years, with the introduction of more effective purine analogues (azathioprine) and in particular anti-TNF-α biologics (Mozaffari et al., 2014), meaning more patients are staying in clinical remission for longer. Due to the complex and diverse nature of IBD, a variety of treatments are used singly and in combination. This enables patients who fail on one treatment to be switched or escalated onto a more potent treatment.

1.6.1   Feeds

Exclusive enteral nutrition (EEN) is generally used as a first treatment after diagnosis of paediatric IBD to induce remission. It consists of the patient exclusively gaining nutrition through a fibre-free, liquid feed derived from hydrolysed milk protein (polymeric), or from individual amino acids (elemental) to reduce inflammation of the GI tract and allow mucosal healing to occur. While the precise mechanism of action of EEN is unknown, it has been shown to be a reliable and potent inducer of histological remission in paediatric IBD (Nahidi et al.,

2014). The effects of EEN are greater than simply improving the nutritional status of the patient; a study following the levels of inflammatory markers (erythrocyte sedimentation rate (ESR), interleukin (IL)-6, C-reactive protein (CRP))showed they decreased before any improvements in nutritional or growth status were observed in paediatric CD (Bannerjee et al., 2004). It has been hypothesised that the increased effectiveness of EEN on paediatric CD compared to UC is due to the stimulation of the anti-inflammatory cytokine transforming growth factor (TGF)-β, which instead is reduced in UC upon EEN treatment (Wedrychowicz et al., 2011). However, the actual mechanism of action of EEN is not wholly understood. While increased microbiome diversity is protective against IBD (discussed above), EEN in fact reduces the diversity of the microbiome in CD (Gerasimidis et al., 2014). The current consensus is that the effects of EEN are multi-factorial, and include elimination of fibre preventing further mechanical damage to the GI tract, reduction in diversity of the microbiota "resetting" the organisms present in the gut (Gerasimidis et al., 2014), restoration of intestinal barrier function (Miner-Williams and Moughan, 2016) and possible direct manipulation of the cytokine milieu (Wedrychowicz et al., 2011).

While virtually side-effect free, EEN does have one potential drawback that is possibly enhanced when used in paediatrics. Adhering to the exclusive diet for up to 12 weeks is difficult, with the feeds tasting unpleasant and social pressures at school compounding the temptation to break with diet adherence. This is one of the reasons EEN is used only as an induction therapy, and other pharmacological agents are used for maintenance of remission.

1.6.2 Corticosteroids

The main corticosteroid used in the treatment of IBD is prednisone, which when metabolised releases the active molecule prednisolone, a synthetic analogue of cortisol and is used for its immune-suppressive properties. Prednisolone acts by binding cytosolic glucocorticoid receptors, changing gene expression within the cell and promoting an anti-inflammatory state (Stahn et al., 2007). This mechanism is utilised when treating IBD, and corticosteroids are used

short-term to induce remission in IBD, but have no effect on rate of relapse (Ford et al., 2011a). This combined with the many and varied side-effects of long-term corticosteroid use mean that once remission has been achieved, the patient is weaned off the treatment and put on another to maintain remission. Side effects can include diabetes mellitus, psychiatric complications, increased fracture risk and hypertension (Curkovic et al., 2013). Due to their side-effects, corticosteroids are considered a less than ideal drug, such that the efficacy of other therapies are sometimes measured by the outcome of reduced corticosteroid use (Walsh et al., 2014).

1.6.3   Methotrexate

Methotrexate acts as a competitive inhibitor for the enzyme dihydrofolate reductase (Rajagopalan et al., 2002). This enzyme catalyses the conversion of dihydrofolate into the active tetrahydrofolate, used for the *de-novo* synthesis of thymidine and purine bases, which are required for DNA synthesis and therefore cell division. It is this action that makes methotrexate in high doses a chemotherapeutic agent for cancers (Du et al., 2012; Meyer et al., 1950). At a lower dose in IBD, it is thought that methotrexate works in a similar way to the mechanism of action in RA, namely "reducing cell proliferation, increasing the rate of apoptosis of T cells, increasing endogenous adenosine release, altering the expression of cellular adhesion molecules and influencing production of cytokines, humoral responses and bone formation" (Wessels et al., 2008). In paediatric IBD, methotrexate is used singly and in combination with biologics to treat steroid-dependent CD, acting as an overall immuno-"prophylactic" (Herfarth et al., 2016).

1.6.4   5-aminosalicylates

5-aminosalicylate (5-ASA) is the active agent of the drugs mesalazine and sulfasalazine, used as anti-inflammatory drugs in IBD, though mainly in UC (Criscuoli et al., 2013). The original formulation, sulfasalazine, consisted of 5-ASA bound by a diazo bond to sulfapyridine, a therapeutically inactive moiety (Baron et al., 1962). Once it was discovered that the significant

levels of side-effects seen with sulfasalazine were due to the sulfapyridine moiety (Azad Khan et al., 1977), new preparations containing only 5-ASA were formulated, modified to prevent rapid absorption through the jejunum. It can be taken orally for the treatment of CD, and as a rectal suppository for the treatment of UC. 5-ASA is predominantly used to treat mild-to-moderate UC (Feagan and Macdonald, 2012a), as well as maintaining remission in UC (Feagan and Macdonald, 2012b). As well as reducing inflammation in the GI tract, large epidemiological studies have shown 5-ASA to reduce the risk of colorectal cancer in IBD patients (van Staa et al., 2005). Recently however, combination therapy of 5-ASA with azathioprine (see below) has been shown to potentially increase myelotoxicity (Gao et al., 2012).

The mechanism of action of 5-ASA is unknown but thought to be multi-factorial, including "inhibition of prostaglandin and leukotriene synthesis, free radical scavenging, immunosuppressive activity, impairment of leukocyte adhesion and function, inhibition of cytokine synthesis and signal transduction including the key transcription factor of inflammation NF-κB and MAP kinases" along with promoting wound-healing in a TGF-β independent manner (Baumgart et al., 2005).

1.6.5 Purine Analogues

The main purine analogue used to treat IBD is azathioprine, which when metabolised along with the similar drug 6-mercaptopurine (6-MP), release pharmacologically active 6-thioguanine nucleotides. These nucleotides prevent clonal expansion of lymphocytes, acting as an immune-suppressive agent, by inhibiting the action of purine-metabolising enzymes and preventing DNA synthesis (Allison et al., 1977; Dubinsky, 2004). Azathioprine is used in IBD as a steroid-free maintenance therapy, and is one of the main immunomodulatory drugs used for the treatment of IBD.

There is an important consideration when using azathioprine, which is one of the few examples of pharmacogenetics being used in routine clinical care. The enzyme thiopurine methyltransferase (TPMT) converts 6-MP into an inert metabolite, and its action prevents the

build-up of cytotoxic metabolites that can cause leukopenia, amongst other adverse drug reactions (Hindorf and Appell, 2012). Different polymorphisms within the general population affect the efficacy of this enzyme, and therefore the rate of inactivation of 6-MP. It has been shown that TPMT monitoring and subsequent dose reduction in hetero/homozygotes for TPMT variants reduces the incidence of adverse drug reactions to azathioprine 10-fold in IBD (Coenen et al., 2015). The close monitoring of 6-thioguanine nucleotides in patients is an additional requirement in order to properly titrate the dose into an efficacious range (Hindorf and Appell, 2012).

1.6.6   Biologics

One of the most recently developed categories of drug used in the treatment of IBD are biologics. These consist of antibodies that have been raised to a specific antigen associated with IBD, and accelerate the neutralisation and clearance of their target by opsonisation and complement activation (Kaymakcalan et al., 2009). The first such drug to be approved for use in IBD was infliximab, a chimeric mouse/human monoclonal antibody that binds to TNF-α, forming a complex that is then recognised and cleared by the immune system (Liang et al., 2013). By reducing levels of TNF-α in the patient, infliximab has been shown to reduce the number of CD68^{+} macrophages and mucosal IL-17A expressed in the intestines of IBD patients, and that these reductions strongly correlated with mucosal healing (Caprioli et al., 2013). This suggests that by reducing TNF-α, infliximab is reducing the overall inflammatory milieu within patients.

Meta-analyses have demonstrated the efficacy of infliximab in inducing remission in CD and UC, and preventing relapse of moderate to severe UC (Ford et al., 2011b), and it is used in the treatment of IBD refractory to other treatments. Monitoring of circulating levels of infliximab is very important, as not only do low trough levels (the lowest level infliximab reaches between doses) of circulating infliximab reduce its efficacy (Imaeda et al., 2014), but it also increases the likelihood of the patient raising an immune response to infliximab and developing antibodies

to it (Baert et al., 2014). While effective for a proportion of patients long term, a significant number of patients eventually fail to respond due to the above reasons (Steenholdt et al., 2015).

A fully humanised version of infliximab, adalimumab, has been developed, which has similar efficacy and safety profiles. However, one advantage gained from this extra treatment is that it has been shown to be safe and effective in the majority of cases to treat paediatric CD patients that have failed on infliximab, giving these patients a potential life-changing treatment when others have failed (Fumery et al., 2015). The monoclonal antibody golimumab and pegolated antibody fragment certolizumab pegol are both also approved biologics for IBD that target TNF-α, but will not be elaborated on as no patients studied in this thesis were treated with them.

Other biologics rarely used in paediatric IBD are natalizumab (Singh et al., 2016a) and vedolizumab (Singh et al., 2016b), both of which target the α4-integrin and reduce the ability of leukocytes to extravasate and migrate. This reduces the build-up of leukocytes, and therefore inflammation within the GI lumen in IBD. There is little data on their use in paediatric IBD, but small studies have shown their efficacy in patients who have failed anti-TNF-α therapy (Singh et al., 2016a; Singh et al., 2016b). A potential safety concern with anti-α4-integrin therapies is that they can provoke multifocal leukoencephalopathy in patients that are John Cunningham Virus positive, which combined with the limited data in paediatric IBD means they are rarely used.

Overall the potential of biologic therapy has barely been utilised, and as further effectors of IBD pathogenesis are discovered, the use of biologics to modify them could be an important part of the future therapeutic arsenal.

1.6.7 Surgery

For those patients whose disease is resistant to treatment, or those who suffer complications such as bowel strictures or intestinal perforations, the last line of treatment is surgical intervention, often as a "salvage" operation (Solina et al., 2016). In the case of CD, the most common procedure is ileocolic resection, removing a section of affected intestine and re-joining the two ends (Dignass et al., 2010). Surgical techniques have progressed from this being an open surgery to being performed laparoscopically, with corresponding shorter hospital stay and fewer complications (Milsom et al., 2001). The most common procedure in UC patients is proctocolectomy with ileal pouch-anal anastomosis, with the removal of the terminal bowel and formation of a stoma (Kessler et al., 2011). This is also now performed normally laparoscopically, and due to UC localisation to the terminal bowel can greatly reduce inflammatory symptoms. This must be balanced against the morbidity and reduction in quality of life of living with an ileostomy.

1.7 Biomarkers of IBD

Beyond visible inflammation of the GI tract when viewed by endoscopy, the development of biomarkers for the non-invasive assessment of IBD severity has not been very successful (Bennike et al., 2014). There are no biomarkers used in the clinic for the differentiation between CD and UC, with diagnosis still based on symptoms and clinical features. Currently in the clinic most markers used are for general inflammation, such ESR and CRP (Fengming and Jianbing, 2014). These biomarkers can also be raised by intercurrent infections or other diseases, making them non-specific and difficult to interpret without full clinical information.

The only biomarker validated for the assessment for GI inflammation specifically is the level of faecal calprotectin (Waugh et al., 2013). Calprotectin is a complex of S100A8 and S100A9 proteins, and in the presence of calcium can sequester manganese and zinc, giving it antimicrobial properties (Lehmann et al., 2015). Calprotectin makes up a large proportion of the neutrophil cytosol, and is secreted by neutrophils upon activation (Stríz and Trebichavský,

2004). Its presence in faeces therefore is an indicator of inflammation within the GI tract. While a useful biomarker, faecal calprotectin does show large intra and inter-individual variability (Kristensen et al., 2016), highlighting the care with which values should be interpreted. However it remains an important positive marker for IBD, helping to exclude non-inflammatory diseases such as irritable bowel syndrome (IBS) (Chang et al., 2014).

Anti-*Saccharomycese cerevisiae* antibodies (ASCA) and perinuclear antineutrophil cytoplasmic antibodies (pANCA) have been investigated as possible IBD biomarkers due to their raised values correlating with having the disease. While they have been useful with stratifying the risks of disease phenotype and differentiating CD and UC patients, they have shown limited utility for making a definitive diagnosis of IBD (Zhou et al., 2016).

Investigation into possible novel biomarkers for IBD have shown the utility of serum levels of matrix metalloprotease (MMP)-9, and IL-22 (Faubion et al., 2013) in discerning IBD. Additionally, there are novel attempts using proteomics to find unique protein signatures in inflamed intestinal mucosa, and then looking for these signatures in easier-to-reach tissues such as the blood (Roda et al., 2010; Bennike et al., 2014). The hope is that using these techniques, novel biomarkers can be found that help differentiate CD and UC, and help monitor disease activity to inform treatment.

1.8 IBD Co-morbidities

In addition to the known primary morbidities of IBD, a small but significant increase is seen in the incidences of cancer, with a relative risk of 1.5 and incidence of 0.53% per patient year of follow-up (Algaba et al., 2013). The majority of the increased cancer incidence is due to colorectal carcinoma; the mechanism of this is thought to be due to inflammation-induced carcinogenesis (Romano et al., 2016). Beyond inflammation-induced cancers, IBD treatments can also cause cancers, with azathioprine use associated with increased incidence of lymphoma and skin cancers (Pasternak et al., 2013). In addition, infliximab use has been associated with increased risk of melanoma (Biancone et al., 2015). This risk must be balanced

with the decreased morbidity of maintaining IBD remission, which in turn reduces colorectal cancer risk (Dyson and Rutter, 2012).

As well as increased risk of cancer, cardiovascular risks are starting to be investigated in relation to IBD, as detailed in the next section.

1.9 Cardiovascular risk in IBD

In addition to the known primary morbidities of IBD, emerging but as yet limited evidence suggests an increased life-time risk of coronary artery disease (Gandhi et al., 2012), myocardial infarction, stroke, and cardiovascular death (Kristensen et al., 2013; Fumery et al., 2014). These increases in cardiovascular risk additionally correlated with disease severity. This is evident even when accounting for conventional cardiovascular risk factors such as body mass index (BMI), smoking status and lipid profile, as well as the known increased thromboembolic risk associated with being a hospital inpatient. These risks could be secondary to lifestyle changes brought by the disease, or a direct result of the systemic inflammation associated with IBD. While cardiovascular risks have been shown to be increased in active disease, it is not yet known if these increased risks are present in well-managed patients, or only manifest in more severe cases.

Strokes were shown in a meta-analysis to have moderately increased risk of occurring in IBD patients (OR 1.18, 95% CI 1.09-1.27), particularly in those under 40 years old (Singh et al., 2014), as well as ischaemic heart disease (OR 1.19, 95% CI 1.08-1.31), particularly in women (OR 1.26, 95% CI 1.18-1.35). Both of these phenomena occur due to atherosclerotic changes in the arteries, which eventually restrict blood flow enough to cause ischaemia either in the brain (ischaemic stroke) or the heart (myocardial infarction). Even when conventional risk factors for these changes were accounted for, both of these phenomena show significant increased risk of occurring in IBD patients. Due to the lower incidence of arterial cardiovascular events in children and a paucity of data, it is not known if this risk is particularly increased in those IBD patients with paediatric onset.

While the increased risk of cardiovascular events in IBD patients is epidemiologically evident, looking for markers of cardiovascular risk in IBD patients has had limited success. A meta-analysis of changes in carotid intima media thickness, a metric for atherosclerotic changes to the arteries, was inconclusive (Theocharidou et al., 2014). However, IBD patients, in particular CD, show increased triglyceride levels, a risk factor for cardiovascular disease. There is a great deal of uncertainty as to the mechanisms of these observed changes in IBD, and require further investigations to probe the potential causes.

1.9.1   Thromboembolic Disease in IBD

Compared to the above discussed cardiovascular diseases, thromboembolic diseases, or diseases of abnormally increased blood clotting, occur predominantly in the veins, though can also be arterial. It is estimated 1 in 12 people will have a thromboembolic event in their lifetime (Bell et al., 2016), carrying with it significant morbidity and risk of mortality. The most common thromboembolic event is deep vein thrombosis (DVT), from which a clot can dislodge and block a pulmonary artery, causing a pulmonary embolism. Having IBD increases the lifetime incidence of venous thromboembolism by 2-4 fold (Tichelaar et al., 2012).

An important feature of thromboembolic risk in IBD is that while the incidence of thromboembolism increases with age, the relative risk in younger patients is much higher than older IBD patients. In a Danish population study, while the overall relative risk for a venous thromboembolic event in IBD patients was raised (RR 2.0, 95% CI 1.8-2.1), when looking at patients under 20 years old, relative risks for DVT (RR 6.0, 95% CI 2.5-14.7) and pulmonary embolism (RR 6.4, 95% CI 2.0-20.3) were much higher (Kappelman et al., 2011). This increase was maintained even when accounting for co-morbidities and medication use, and so can be attributed to a primary consequence of the disease and not, for example, staying in a hospital bed.

The most common and simplest way to reduce thromboembolic risk in a patient is the prophylactic use of an anticoagulant, such as low molecular weight heparin. Indeed, post-

operative venous thromboembolism is common (2-3% of patients) in IBD patients undergoing abdominal surgery, but the risks are poorly addressed with a retrospective study of 7078 patients showing only 0.6% of patients were given an anticoagulant post-discharge (Brady et al., 2017).

1.10   Thrombosis and the coagulation cascade

One of the main determining factors of cardiovascular risk is the thrombotic state of the subject. Thrombosis is formation of fibrin clots in blood, and is under the control of a vast network of pro and anti-coagulative agents (Tanaka et al., 2009). These closely control the action of the serine protease thrombin, which cleaves fibrinogen and causes blood clots to form (Onasoga-Jarvis et al., 2014). Close control is required, as a pro-thrombotic state can cause excessive clotting and potential ischaemia, whereas an anti-thrombotic state increases the risk of uncontrolled bleeding. It could be that IBD is potentially causing a pro-thrombotic state, causing the increased incidents of cardiovascular disease. The role of tissue factor (TF) and the vascular endothelium is further explored and investigated in Chapter 7.

1.11   Microparticles (MPs)

An increasingly studied feature of cancers and many systemic inflammatory diseases are circulating microparticles (MPs); small extracellular vesicles derived from the cellular plasma membrane of many cell types including platelets, leukocytes and the vascular endothelium (van der Pol et al., 2015).

These are phenotypically distinct from exosomes, vesicles <100nm in diameter that are excreted by cells when a multi-vesicular body fuses with the plasma membrane (D'Souza-Schorey and Clancy, 2012). MPs are also distinct from apoptotic fragments, parts of apoptotic cells >1μm in diameter, released during cell death and breakdown. MPs can also be, and in the majority are, excreted by non-apoptotic cells, whereas apoptotic fragments are derived only from dead cells (Baron et al., 2012). Currently, the mechanisms of MP release are not fully elucidated.

MPs express the surface and membrane proteins of their parent cell, and are also pro-coagulative due to the catalytic action of their enriched phosphatidylserine surface (Owens and Mackman, 2011). There have been limited studies of MPs in IBD, where an increase in adult CD patients has been recorded (Leonetti et al., 2013). In an *in-vivo* murine model, injection of IBD MPs resulted in impairment in endothelium-dependent relaxation (Leonetti et al., 2013). Additionally, MPs isolated from paediatric IBD patients show an increase in thrombin generation, showing a pro-thrombotic tendency (Deutschmann et al., 2013). Taken together, these studies indicate that an impact of IBD pathogenesis on cardiovascular health is likely. At present no information is available on endothelial dysfunction, circulating MPs, cardiovascular health and prothrombotic potential within the same paediatric cohort. In addition, evidence for vascular dysfunction in well-managed patients *i.e.* those in remission or with mildly active disease is unknown.

1.12   Hypotheses and Aims

In this thesis, I explored the hypothesis that chronic, subclinical inflammation in well-managed inactive or mild IBD, continues to promote increased cardiovascular risk by causing vascular endothelial dysfunction. This results in an increase in circulating MPs (a newly established systemic inflammatory mediator (Collier et al., 2013; Eleftheriou et al., 2011)), and increased thrombin generation potentially causing a pro-coagulation state, and increased arterial stiffness.

By investigating MP release, and recruiting paediatric IBD patients to a cross-sectional observation study, we hoped to answer the following questions:

- What are the mechanisms and characteristics of endothelial MP release?

- Do children with IBD show evidence of endothelial injury?

- Do children with IBD show evidence of a microparticle-mediated pro-thrombotic tendency?

- Do children with IBD show evidence of structural arterial disease?

- Do children with IBD show increased circulating pro-inflammatory or vascular injury markers?

Whilst IBD is rarer in children than in adults, studying paediatric IBD provides important advantages when considering cardiovascular risk. These include a reduced impact of important confounding variables such as smoking, hypertension, diabetes and pre-existing heart conditions. This population choice may also allow detection of vascular changes at the pre-clinical stage, potentially providing a window for therapeutic intervention and maximising the effect of any intervention. This is the first study detailing multiple parameters in a single cohort, providing a more comprehensive analysis of cardiovascular risk in paediatric IBD.

2. Methods

2.1 Cell Culture Studies

2.1.1 Cell Culture Reagents

All plastics, which include 25cm^2, 75cm^2 and 175cm^2 tissue culture flasks, 6 and 12 well plates, pipettes and polypropylene Eppendorf tubes were purchased from Fischer Scientific, Leicestershire, UK. Cell culture reagents were purchased from Invitrogen, Paisley, UK, unless otherwise stated.

2.1.2 Vascular Endothelial Cell Lines

Human umbilical vein endothelial cells (HUVECs) are primary large-vein vascular endothelial cells isolated from umbilical cords using collagenase to detach them from the smooth muscle of the largest vein. When grown in culture, HUVECs grow relatively slowly (around 5 days from initial seeding to full confluency) and do not easily grow beyond passage 6, obtaining more fibroblast morphology as passage number increases.

EA.hy926 cells are an immortalised hybrid of HUVECs and the lung epithelial carcinoma cell line A549 (Edgell et al., 1983). They have endothelial morphology (Figure 2.1), display endothelium specific Weibel-Palade bodies (Edgell et al., 1990) and show angiogenesis behaviour (Bauer et al., 1992).
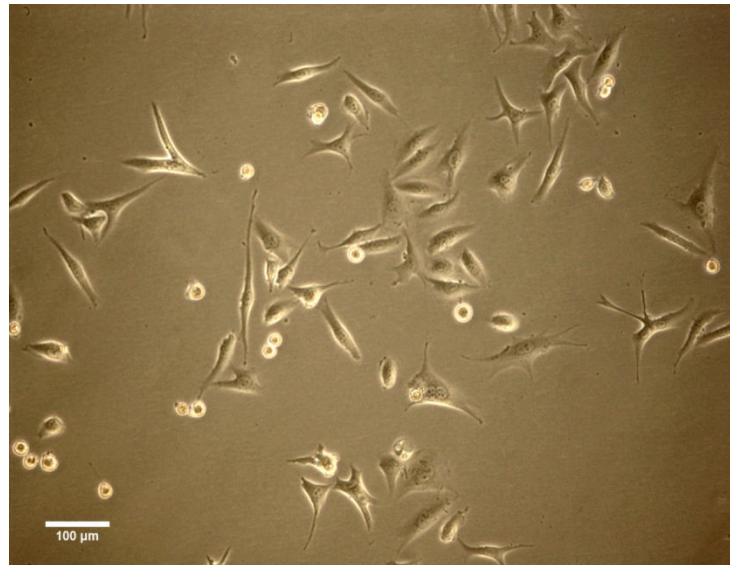
Figure 2.1: EA.hy926 cells at 50% confluency

2.1.3   Counting Viable Cells

Cells in Suspension

10μL of homogenous cell suspension was mixed with 10μL of 0.4% trypan blue (Sigma, Poole,

UK). 10μL of this mixture was placed onto a haemocytometer (Hawksley & Sons Ltd., West

Sussex, UK) counting chamber, and viable cells were counted as those that did not take up the

dye, viewed at a magnification of 40x (Zeiss Microscope, Germany). Viable cells were counted

in 100 squares and the cell concentration was calculated as:

Cells in 100 squares $\div 4$ x dilution factor (2) = Number of cells x 10^4/mL.

Adherent Cells

Media was removed and cells were washed twice with sterile phosphate buffered saline (PBS),

before addition of 1mL per 25cm^2 culture flask of Accutase® solution. After 3-5min, when cells

appeared spherical under the microscope, Accutase® activity was inactivated by addition of

MCDB- 131 + 2mM glutamine culture medium, supplemented with 20% foetal calf serum

(FCS), 100 units/mL penicillin, 100μg/mL streptomycin and 0.25 μg/mL Fungizone®. This media

is referred to as MCDB complete henceforth. The resulting cell suspension was processed as

per *cells in suspension*. All other cell types were treated as above, but Accutase® was replaced with Trypsin (Sigma, UK) solution.

2.1.4   Cell Passage

HUVECs and EA.hy926 cells were regularly checked under the microscope for contamination and morphological changes signifying differentiation and growth. Cells in suspension were also regularly counted as described above to ascertain cell density. When adherent cells reached a confluency of >80%, they were passaged. Adherent cells were resuspended as per 2.1.3, and the cell suspension was centrifuged at 1500rpm for 5min at room temperature (RT; Rotina 46R centrifuge, Wolf Laboratories, UK). The subsequent cell pellet was resuspended in 1mL MCDB complete media, and seeded at an appropriate density in $25cm^2$ or $75cm^2$ culture flasks.

2.1.5   Cell Freezing

For long-term storage, cells were Accutase treated if adherent as per 2.1.3 , centrifuged and then resuspended in media containing 90% FCS + 10% dimethyl sulphoxide (DMSO; Sigma, Poole, UK) and transferred into cryovials at a cell density of $1x10^6$ cells/mL (Nunc, Roskilde, Denmark). Vials were quickly placed into a Nalgene Mr Frosty freezing container (Sigma, Poole, UK) filled with isopropanol and frozen at -80°C for 24h. Subsequent long-term storage was in liquid nitrogen.

2.1.6   Cell Thawing from -80°C

Cryovials were removed from -80°C storage and quickly thawed to 37°C in a water bath. 10mL of MCDB complete media at 37°C was added and the cells centrifuged (500 x *g*, 5min, 4°C) to remove the DMSO. Cell pellet was resuspended in MCDB complete media and transferred to a $25cm^2$ tissue culture flask and allowed to grow.

2.1.7   HUVEC Preparation and Maintenance

The human umbilical vein derived endothelial cell line (HUVEC) was used as a vascular endothelium model. Each donor umbilical cord was treated as a separate experiment to highlight variability between individuals. Cords were collected by midwives at Middlesex Maternity Ward and stored at 4°C in RPMI culture medium, supplemented with 100 units/mL penicillin, 100μg/mL streptomycin and 1.25 μg/mL Fungizone® (Gibco). Upon arrival in the laboratory, cords were checked for structural integrity, and then sealed at either end with surgical clamps. The umbilical vein was filled with 10-15mL RPMI culture media using a 20mL syringe and 127mm Kwill filling tube (Smiths Medical), and then flushed through by removing the bottom clamp. The vein was then re-clamped and filled with 10-15mL of type II collagenase at 1g/L in RPMI, and incubated at 37°C for 10min, before being gently massaged and the contents flushed into a 50mL falcon tube. The cord was then flushed through with 5mL of 0.22μm filtered FCS into the same tube. The tube was centrifuged at 1500rpm for 5min at RT (Rotina 46R centrifuge, Wolf Laboratories, UK) and the resultant pellet was resuspended in MCDB complete media. The cells were inoculated into flasks at 1 umbilical cord per 25cm^2 flask, and maintained at 37°C and 5% CO_2 in a humidified incubator (Galaxy CO_2 Incubator, Wolf Laboratories Ltd., Pocklington, UK).

HUVECs were maintained in 25cm^2 flasks, with the culture media changed every 2 days. Once cells were 90% confluent, they were passaged (as per 2.1.4) into 6-well or 24-well plates at a density of 1 confluent cord per plate. HUVECS prepared from umbilical cords in the laboratory were not used beyond the first passage.

HUVECs were also bought commercially as a vial of 1x10^6 cells, pooled from 5 donors (Sigma, UK). These were thawed and maintained as above, and not used beyond passage 4, with passage 1 being initial thawing.

2.1.8 EA.hy926 Maintenance

EA.hy926 cells were maintained in Dulbecco's Modified Eagle (DMEM) culture medium, supplemented with 10% v/v 0.25μm sterile-filtered FCS, 100 units/mL penicillin, 100μg/mL streptomycin and 1.25μg/mL Fungizone®. This medium will in future be referred to as DMEM complete. When passaging, cells were seeded at a density of $2x10^3$ cells per cm^2 surface of culture container. Cells were used from passage number 72 to 82.

2.1.9 Cytokine Stimulation and inhibitor dosing of HUVECs

Prior to stimulation, media from fully confluent HUVECs in 6-well or 24-well plates was replaced with 0% FCS MCDB-131 + 2mM glutamine media, supplemented with 100 units/mL penicillin, 100μg/mL streptomycin and 0.25 μg/mL Fungizone®. This media is referred to as serum-free MCDB henceforth. Cells were incubated for 2h then the media discarded and replaced with 10% FCS MCDB-131 + 2mM glutamine media, supplemented with 100 units/mL penicillin, 100μg/mL streptomycin and 0.25 μg/mL Fungizone®, and the cytokine or inhibitor added directly to the fresh media. Inhibitor stocks that were dissolved in DMSO were compared to a control condition that contained media with the same concentration of DMSO. After incubation, the culture media was aspirated, transferred to a 1.5mL conical microcentrifuge tube and spun at 3000g for 5min. The supernatant was transferred to a fresh 1.5mL tube and frozen at -80°C for future analysis.

2.1.10 Cytokine stimulation and inhibitor dosing of EA.hy926 cell line

Prior to stimulation, cell growth media was replaced on fully confluent cells with 1mL per well DMEM + 0.1% FCS for 1h in a 37°C, 5% CO_2 incubator. Media was then replaced with fresh DMEM + 0.1% FCS with the experimental cytokine or inhibitor pre-mixed to working concentration. All cytokines were obtained as lyophilised recombinant proteins from Peprotech. Cytokines or inhibitor stocks dissolved in DMSO were run concurrently with a control experiment, consisting of the same media with the same concentration on DMSO, free of the cytokine/inhibitor. Stocks dissolved in culture media were run concurrently with a

control experiment incubated in only media. After incubation at 37°C 5% CO_2 for the experimental time period, supernatants were aspirated by 1mL pipette and transferred to 1.5mL conical tubes then spun (5000 x g, 5min, 20°C) to remove cellular debris. The subsequent supernatants were transferred to fresh tubes by pipette and immediately frozen at -80°C until analysis.

2.1.11 Human Neutrophil isolation and stimulation

15mL of healthy adult volunteer blood was taken from the median cubital vein, with tourniquet applied, using a 21-gauge needle with butterfly extension, and added to a 50mL tube containing heparin to a final concentration of 15 U/mL. 15mL of sterile-filtered 3% v/v dextran solution was added to the tube, and left to settle for 40min or until erythrocytes had fully sedimented. The buffy coat was removed with a plastic Pasteur pipette and gently layered equally into two 15mL tubes, both filled with 5mL Ficoll®-Paque (GE Healthcare, UK). The tubes were centrifuged (600 x g, 20min, 20°C), then using a Pasteur pipette, all layers above and including the Ficoll were removed. The remaining layers were carefully disrupted with 1mL ddH$_2$O, then immediately transferred to a 50mL falcon tube and suspended in 45mL Hank's Buffered Saline Solution (HBSS), without Ca^{2+} or Mg^{2+} ions. This tube was centrifuged (500 x g, 5min, 20°C), the supernatant removed and the wash step repeated to remove any remaining un-lysed erythrocytes. The final pellet was suspended in RPMI to a cell density of $2x10^6$/mL, determined by cell counting on a haemocytometer.

The neutrophil preparation was immediately stimulated with phorbol 12-myristate 13-acetate (PMA) at 2ng/mL for 2h at 37°C, 5% CO_2. After stimulation, the tube was centrifuged (500 x g, 5min, 20°C) and the supernatant frozen at -80°C in 200µL aliquots for use as a neutrophil microparticle control in flow cytometry.

2.1.12 Plasma preparation from Whole Blood of Adult Donors

MP-free Plasma

Blood was drawn from the median cubital vein of healthy adult donors, with tourniquet applied to upper arm, using a 21-gauge needle with butterfly extension, into a 20mL syringe. The first 5mL of blood was discarded to remove contaminating endothelial cells dislodged from the puncture site. The blood was decanted into 50mL falcon tubes containing sodium tricitrate to a final concentration of 0.106M. Within 30min the collected blood was transferred to 1.5mL conical tubes and centrifuged at 5000g for 5min. The supernatant was carefully decanted into a second tube and the centrifugation repeated. The supernatant was decanted into a fresh tube, and then centrifuged at 20,000g for 1h at 4°C. The supernatant was carefully aspirated until 20-40μL was left in the tube and decanted into a new 1.5mL conical tube, and frozen at -80°C for further use.

2.1.13 Microparticle (MP) Isolation

Stored cell supernatants or platelet poor plasma (PPP) were thawed quickly in a 37°C water bath, and then centrifuged at 20,000g for 1h at 4°C. The supernatant was carefully aspirated until 20-40μL was left in the tube, and the resultant pellet (usually invisible) was resuspended in 1x Annexin V binding buffer (BD Biosciences) for flow cytometry, or resuspended in MP-free plasma (MPFP) for thrombin generation analysis.

2.1.14 Microparticle Staining for detection and quantification by Flow Cytometry

MPs were prepared in Annexin V binding buffer as per 2.1.13, and then all antibodies needed for staining were prepared at 50x or 20x final dilution in a total of 10μL, using 1x Annexin V binding buffer to dilute as necessary (Table 2.1). Monoclonal antibodies for cell-surface receptors, and Annexin V for phosphatidylserine (PS), were conjugated to fluorescent molecules (Table 2.1). All antibodies were titrated to ascertain their optimal working dilution. All panels were tested on control MPs from a single healthy adult donor before use in experiments.

40μL of MP suspension was then added to 10μL of the antibody preparation in polypropylene 96-well plates. The samples were covered with a plate sealer and aluminium foil, and placed on an orbital shaker at 500rpm at RT for 30min. 150μL of 1x Annexin V binding buffer was added to each sample, which were stored at 4°C protected from light until analysed.

Probe	Target Name	MP type	Isotype	Fluorochrome Conjugate	Working Dilution (40μl sample)
Annexin V	Phosphatidylserine	All	N/A (Ca^{2+} free)	BV421, FITC, PE	1 in 50
CD14	LPS co-receptor	Monocyte	IgG2a	BV605	1 in 50
CD42a	Glycoprotein IX	Platelet	IgG1	FITC	1 in 50
CD54	ICAM-1	Endothelial (non-specific)	IgG1	PE	1 in 50
CD62e	E-selectin	Endothelial (activation)	IgG1	PE	1 in 50
CD66b	CEACAM-8	Neutrophil	IgG1	PerCP-Cy5.5	1 in 20
CD11b	Integrin Alpha M	Neutrophil	IgG1	PerCP-Cy5.5	1 in 50
CD142	Tissue Factor	Thrombotic	IgG1	APC	1 in 20
CD144	VE-cadherin	Endothelial	IgG1	PE-Cy7	1 in 20

Table 2.1: List of markers used for lineage-specific MP detection by flow cytometry

2.1.15 Flow Cytometry analysis

All cell culture studies were run on a FACSCalibur flow cytometer (BD, UK), using the attached plate reader module. Samples were acquired at 0.5μL/s for 15s, obtaining roughly 1-3x10^5 events per sample. *Ex vivo* blood MP experiments were run on a LSRII flow cytometer (BD, UK), in polypropylene FACS tubes. Samples were acquired on the low flow speed setting for 30s, obtaining roughly 1x10^5 events per sample.

In order to enumerate the MPs and control for variations in flow rate in the flow cytometers, a suspension of 3μm diameter polystyrene beads (Sigma, UK) was run in addition in every analysis. The beads were provided in a stock at 6.667x10^9/mL. 6μL of bead stock was diluted in

2mL sterile filtered PBS, giving the working solution (2×10^7/mL). 10μL of working solution was diluted in 190μL sterile filtered distilled H_2O, giving a total of 200,000 beads. This was acquired by the flow cytometer with the same parameters as the samples, and run in triplicate in the same run as sample analysis (Figure 2.2B). From this, a multiplication factor was calculated and applied to the number of MPs acquired in each sample. This method circumvented the need to acquire the whole of each sample which would take unfeasibly long. The equation to calculate the multiplication factor was:

$$No. of\ MPs\ in\ sample = \frac{200,000}{Mean\ 3\mu m\ bead\ count}\ x\ 5$$

1μm diameter polystyrene beads (Sigma, UK) were used to define the MP gate, with anything classed as a MP if it showed side scatter less than these beads. 3μL of the bead stock was diluted in 2mL sterile filtered PBS, then 10μL of this working solution was diluted in 190μL sterile filtered PBS. This sample was acquired for 15-20s (Figure 2.2A). The same protocol was performed using 0.3μm diameter polystyrene beads to show the size resolving capability of both flow cytometers used (Figure 2.2C,D).
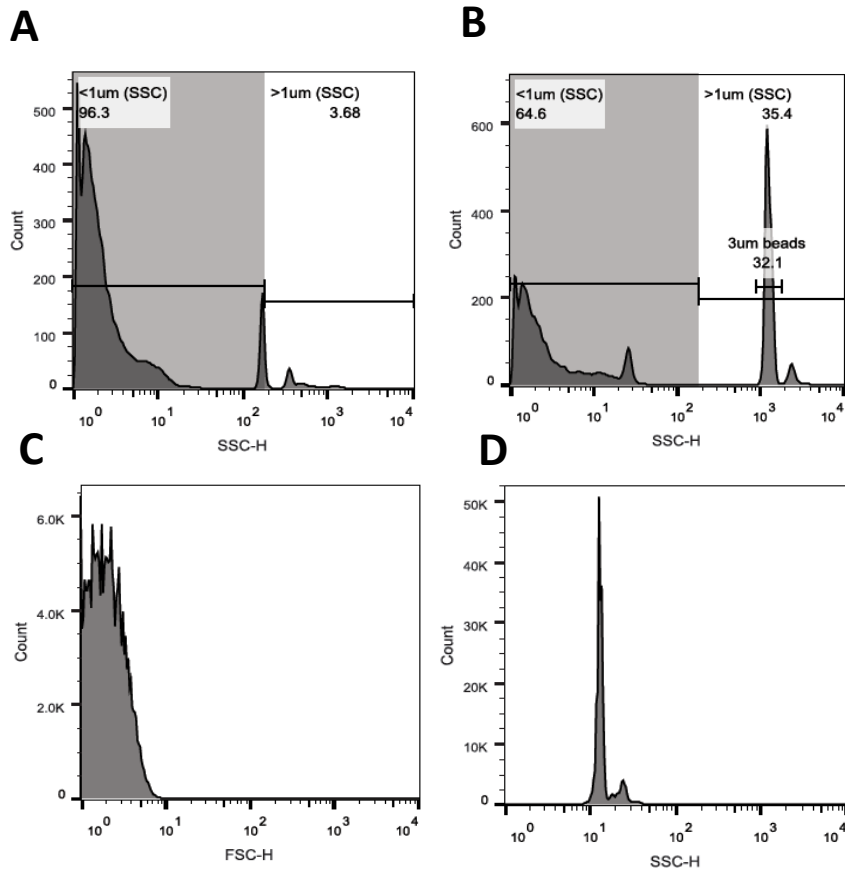
Figure 2.2: Size resolution and gating strategies on FACSCalibur for Microparticle (MP) analysis

*Different diameter (0.3μm, 1.1μm and 3μm) polystyrene beads were run through the FACSCalibur flow cytometer. (**A**) 1.1μm beads helped define a size gate for MP analysis; (B), 3μm beads were used for enumeration calibration. (C) 0.3μm beads were visualised by forward scatter (**D**) and side scatter highlighting the difference in size resolution between the two parameters.*

In order to enumerate MPs on the LSRII flow cytometer for the 7 marker panel, the same bead strategies as above were used. 1.1µm beads (Figure 2.3A) were used to define a size gate, with 3µm beads (Figure 2.3B) used to enumerate. All samples were run on the lowest flow setting the instrument allowed to reduce particle coincidence, and all samples including beads were run for 60s. 3 separate 3µm bead preparations were run and a mean bead count was taken from all 3 to use in enumeration calculations. The lowest size setting was defined by a threshold set on side scatter (SSc) of 500, with 0.3µm beads still being visible as a peak, which was not visible on forward scatter (FSc) (Figure 2.3C,D).

Negative gates for the 7-marker panel were defined using isotype controls conjugated to the relevant fluorophore. 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.
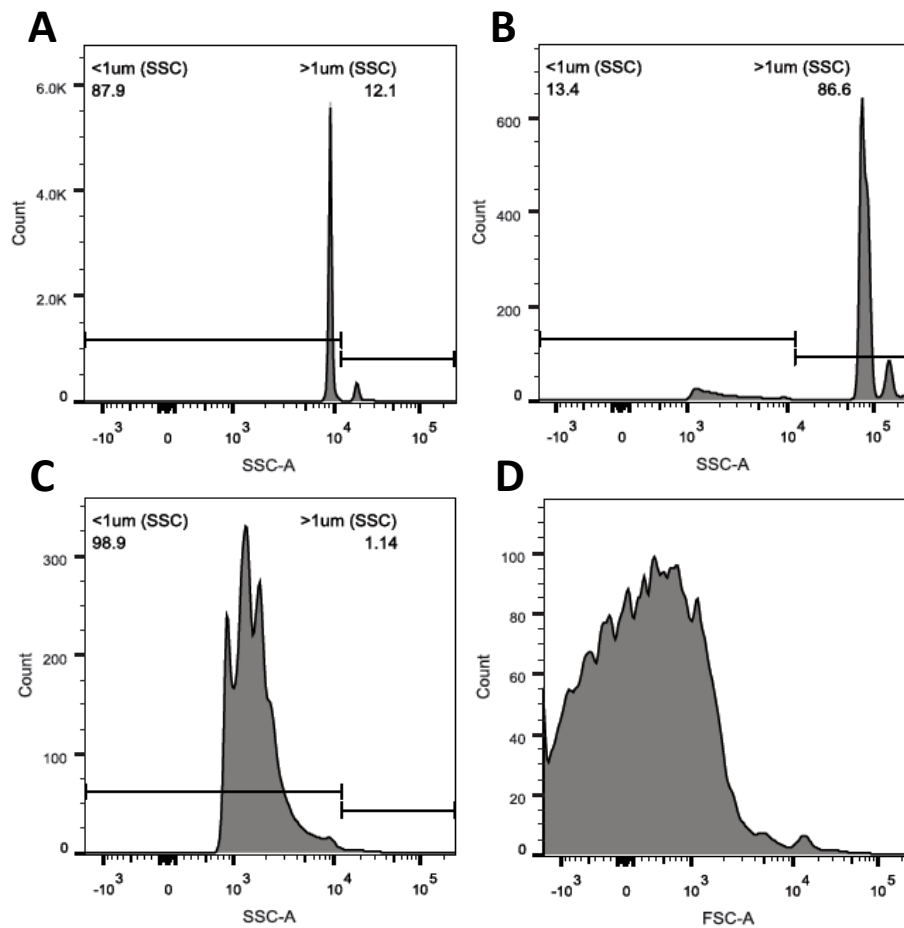
Figure 2.3: Size resolution and gating strategies on LSRII for MP analysis

*Different diameter (0.3μm, 1.1μm and 3μm) polystyrene beads were run through the BD LSRII flow cytometer. **(A)** 1.1μm beads helped define a size gate for MP analysis; **(B)** 3μm beads were used for enumeration calibration. **(C)** 0.3μm beads visualised by side scatter and **(D)** forward scatter highlighting the difference in size resolution between the two parameters.*

2.1.16 Interleukin (IL)-8 Enzyme-linked Immunosorbant Assay (ELISA)

To determine the concentration of IL-8 in the supernatant of EA.hy926 cells, supernatants were harvested and transferred to a 1.5mL microcentrifuge tube. Supernatants were spun in a centrifuge at 5000g for 5min to remove cell debris, transferred to a fresh 1.5mL tube and frozen at -80°C before analysis.

A clear, flat-bottomed 96 well plate (NUNC, UK) was coated with 100µL per well of anti-human IL-8 capture antibody (Peprotech, USA) diluted to 0.5µg/mL in PBS pH 7.4, covered with a plate sealer and left overnight at room temperature. Wells were washed 4 times with 300µL per well with PBS pH 7.4 + 0.05% v/vTween-20 (Sigma, UK), hitherto known as wash buffer, and then blotted dry on absorbent paper. 300µL per well of PBS pH 7.4 + 1% w/v BSA (Sigma, UK) was added and incubated for 1h at room temperature, and then wells were washed 4 times in wash buffer as above. A standard curve was prepared by diluting human IL-8 standard (Peprotech, USA) to 1ng/mL in PBS pH 7.4 and then further serially diluting 1 in 2 (200µL of previous diluent added to 200µL PBS) to create a series of 6 standards. 100µL of standards and samples were added to the plate, covered with a plate sealer and incubated for 2h at room temperature. Wells were washed 4 times in wash buffer, and 100µL biotinylated detection antibody (diluted to 0.5µg/mL in PBS) was added, the wells covered with a plate sealer and incubated for 2h at room temperature. Wells were washed 4 times in wash buffer, and 100µL per well of avidin peroxidase (one 5.5µL aliquot diluted 1 in 2000 in PBS pH 7.4 + 0.1% BSA + 0.05% Tween-20 to a total volume of 11mL) was added, covered with a plate sealer and incubated at room temperature for 30min. Wells were washed 4 times with wash buffer, and 100µL per well of ABTS substrate solution (Peprotech, USA) was added then incubated in the dark for 10min. Absorbance was measured at 405nm with a Multiskan EX plate reader (Thermo Electro Corp, UK), and sample concentrations of IL-8 were determined by interpolating a standard curve from standards by 4-parameter logistic regression.

2.1.17 Thrombin Generation Assay

MPs were isolated from 1mL of cell supernatants or 500μL of PPP, and resuspended in 200μL of MP-free plasma (MPFP), derived from a pool of 5 healthy adult donors. Citrated PPP from study subjects was thawed at RT, and MPs isolated from 200μL PPP. MP pellets were resuspended in 100μL MPFP. 40μL of this preparation was added to a well of a black polypropylene 96-well plate. 50μL of a calcium fluorogenic thrombin substrate (0.5 mM of Z-G-G-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). 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 tissue factor (TF) or phospholipids were added, to ensure thrombin generation was solely due to MP activity. Corn trypsin inhibitor was not added, in order to assess the full coagulation pathway including intrinsic activation. 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 endothelial MP generated thrombogram is shown in Figure 2.4.
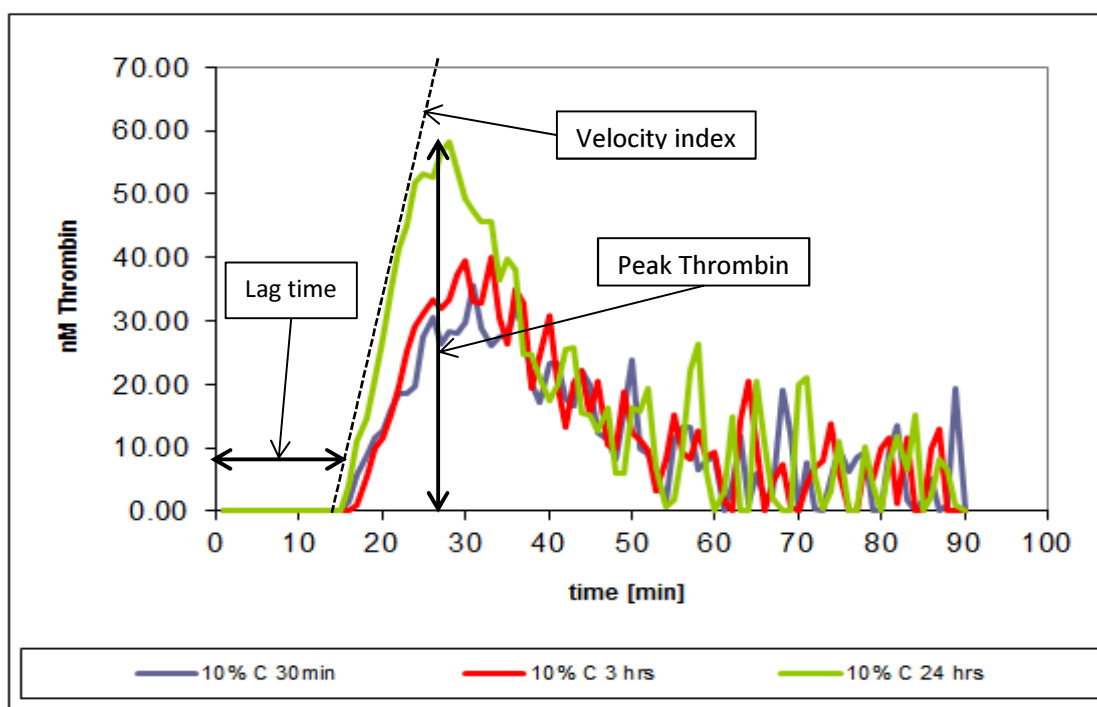
Figure 2.4: A representative thrombogram triggered by endothelial microparticles (MPs)

An example of a thrombogram generated with the thrombin generation assay (TGA). MPs were isolated from the supernatant of human umbilical vein endothelial cells (HUVECs) incubated in 24 well plates for 30mins (blue), 3h (red) and 24h (green). The MP pellet was resuspended in citrated MP-free plasma from 5 pooled healthy donors and a fluorogenic thrombin substrate was added to trace thrombin activity over time. Black arrows show lag time and peak thrombin, with the gradient of the dashed line showing velocity index.

2.2 Clinical Studies

2.2.1 Study Participants

Study participant inclusion criteria:

- Confirmed clinical or endoscopic diagnosis of IBD, classified as Crohn's Disease (CD), Ulcerative Colitis (UC), or IBD-unclassified (IBD-U).

- Aged between 8 and 18 years at time of study.

- Undergoing treatment or disease management at Great Ormond Street Children's Hospital (GOSH) or University College Hospital (UCH).

Study participant exclusion criteria:

- Concurrent infection. However if the infection resolves the participant will be considered at a later date.

- Any concurrent inflammatory disease; for example Coeliac Disease, Rheumatoid Arthritis etc.

- Any genetic cardiovascular disorder. Instances of idiopathic thromboembolism such as stroke or deep vein thrombosis were considered for the study, as these were the precise phenomena I was investigating risk factors for.

All IBD participants were recruited while blinded to disease activity, to prevent bias when assessing circulating endothelial cells (CECs). Disease activity was collected through the Physicians Global Assessment score as part of the ImproveCareNow network (Crandall et al., 2011) after CECs were determined. The parameters collected/calculated/determined for each participant were detailed in Table 2.2:

Parameter	Description
Gender	Recorded on Proforma.
Date of Birth	Found in patient notes.
Disease Classification	Found in Patient notes.
Disease Activity	As classified by Physician's Global Assessment (PGA) within the ImproveCareNow database.
Age at time of investigation	Calculated from date of birth from notes and date of investigation. Expressed in years.
Body Mass Index (BMI) Z-Score	Calculated using height, weight, age and gender compared to the UK90 paediatric growth reference database. Expressed as standard deviations above/below the mean BMI for that gender and age.
Resting Systolic/Diastolic Blood Pressure	Measured by automated oscillometric blood pressure machine on day of investigation.
Lipid Profile	Assessed by GOSH clinical chemistry dept. as a requested test.
Erythrocyte Sedimentation Rate (ESR)	Assessed by GOSH clinical chemistry dept. as part of routine care.
Faecal Calprotectin	Assessed by GOSH clinical chemistry dept. as part of routine care.
Pulse Wave Velocity (PWV)	Carotid-Femoral PWV measured by researcher with Vicorder instrument.
Circulating endothelial Cells (CEC)	Assessed by researcher in the lab.
Platelet poor plasma Microparticle subsets	Assessed by researcher in the lab.
Circulating Cytokine Profile	Assessed by researcher in the lab.

Table 2.2: Parameters collected for each study participant

All study participants were assigned an identification number at random upon recruitment, enabling pseudo-anonymisation of data within the main study database. A single hard copy of the code break, cross-referencing study ID with full name and hospital number was kept in a locked filing cabinet, to allow re-identification of participants if they wished to withdraw from the study. All blood samples were labelled with sample type and study ID only. The study

database was coded and populated within the SPSS 23 software package (IBM, USA) to facilitate future analysis.

2.2.2 Study Participant Venepuncture

All blood samples were obtained concurrently with other blood tests as part of participants' routine care, to prevent additional venepuncture. Blood samples were taken by trained paediatric phlebotomists, from the median cubital vein using a 21-gauge needle with butterfly extension directly into S-Monovette vacutainer tubes (Sarstedt). A tourniquet was used around the upper arm, and at least the first 5mL of blood was used for other routine hospital tests, to avoid contaminating study samples with endothelial cells dislodged from the puncture site. Each study participant had 4 x 1.4mL 3.2% sodium tricitrate, and 3 x 1.2mL 1.6mg/mL K3 EDTA tubes filled, for a total of 9.2mL of blood.

2.2.3 Blood Plasma Preparation and Storage

Blood samples were kept on ice and centrifuged within 1h of being taken. Tubes were kept upright during transport and agitation was minimised. All samples were transferred to 1.5mL conical tubes and centrifuged at 5000g for 5min. Blood plasma was aspirated, taking care to avoid disrupting the sedimented blood cell layer, and transferred to a fresh tube. The centrifugation was repeated, with PPP supernatants transferred to labelled 0.5mL conical tubes and stored at -80°C.

2.2.4 MP Preparation

0.5mL conical tubes of citrated plasma were quickly thawed from -80°C in a 37°C water bath, and immediately upon complete melting transferred to a fresh 1.5mL conical tube. 500µL of PPP was centrifuged (17,000 x g, 1h, 4°C) and the supernatant carefully aspirated with a 200µL pipette, leaving 20µL of undisturbed liquid containing the loose MP pellet. This was resuspended in 500µL Annexin V binding buffer for flow cytometry, or 200µL MP-free plasma (MPFP) for the thrombin generation assay.

2.2.5 Flow Cytometry analysis of Patient Samples

Patient samples were assessed by flow cytometry as per 2.1.14, with single stain control samples derived from healthy donor PPP MPs, and for neutrophil markers, healthy donor neutrophil MPs (NMPs) prepared as per 2.1.11.

2.2.6 Circulating Endothelial Cell (CEC) Enumeration

CECs were enumerated using CD146-coated magnetic bead extraction from whole blood and then positively identified by *Ulex europaeus* Lectin-FITC conjugate staining as previously described in a consensus protocol (Woywodt et al., 2006). The protocol is detailed below:

Preparation of CD146 Beads

Anti-CD146 beads were prepared by transferring 350µL of Dynal M450 mouse pan-IgG beads (Biocytex, UK) into a 5mL tube and fixing the beads on a magnet (Dynamag, UK) for 2min. The liquid was aspirated by pipette and the beads washed with 1mL PBS pH 7.4 + 0.1% w/v BSA (Sigma, UK), known henceforth as bead buffer. This wash step was repeated a total of 3 times, and then the beads were suspended in 950µL bead buffer. 316µL of mouse anti-human CD146 antibody (Miltenyi Biotec, UK) was added, and the mixture was incubated at 4°C on a roller mixer for 2h. This was then washed 3 times in bead buffer as above, and resuspended in 1mL of fresh bead buffer. This bead solution was kept at 4°C and not used beyond 6 weeks of preparation.

CEC Isolation

1mL of participant EDTA whole blood was transferred to a 5mL tube (Eppendorf, UK), and mixed with 1mL PBS pH 7.4 + 0.1% w/v sodium azide (Sigma, UK), 0.6% w/v sodium tricitrate (Sigma, UK) and 0.1% w/v BSA, henceforth referred to as PBAC. To this, 20µL of FcR blocking reagent (Miltenyi Biotec, UK) and 50µL of bead solution was added and the mixture was incubated at 4°C on a roller mixer for 30min. The mixture was fixed on a magnet for 2min, the liquid aspirated by pipette and the beads washed with 1mL PBAC. This was repeated 3 times, the beads were resuspended in 90µL PBAC, and 10µL of 2mg/mL *Ulex europaeus* lectin-FITC

conjugate (Sigma, UK) was added. The beads were protected from light and incubated at RT on a roller mixer for 1h. After incubation, the beads were washed 3 times as above, and resuspended in 200μL PBAC.

2.2.7 *CEC Enumeration*

100μL of bead suspension was pipetted into a Nageotte counting chamber (Hausser Scientific, USA), and CECs were counted on a phase-contrast fluorescence microscope (Leica, Germany). Mercury lamp illumination was passed through a FITC-specific filter cube to illuminate FITC positive cells, and cells were manually counted contained within all 40 lines of the nageotte chamber. CECs were defined as:

- >10μm in diameter

- FITC positive

- Have more than 5 beads attached.

2.2.8 Pulse Wave Velocity (PWV)

Carotid-femoral PWV was measured by Vicorder instrument (Skidmore Medical) as per manufacturer instructions, and in accordance with American Heart Association (AHA) recommendations (Urbina et al., 2009). Participants lay on a bed with the torso angled at 30° from horizontal, with a leg cuff around the right upper thigh and neck pad over the right carotid artery. Distance was taken as the summation of the distance from the centre of the leg cuff to suprasternal notch, and the distance from suprasternal notch to the centre of the neck pad. The cuffs were inflated and the time delay in detecting systole between the cuffs was used along with the distance between them to calculate PWV in m/s.

3 Mechanisms of Microparticle Release

3.1 Introduction

3.1.1 Microparticles

Microparticles (MPs) are sub-micron vesicles derived from the plasma membrane of most cell types. They are produced by plasma membrane blebbing, where the plasma membrane has a localised outward bulge, but the precise mechanisms of release are unknown. They express surface markers of their parent cell that enable deduction of their cell of origin, and can contain proteins (Palmisano et al., 2012; Peterson et al., 2008) and nucleotides, with functional microRNA (miRNA) shown to be transferred within MPs (Alexy et al., 2014). MPs are easily absorbed into the plasma membranes of other cells via endocytosis (Dasgupta et al., 2012) and so exist in dynamic equilibrium in circulation. They are produced in significant numbers by activated platelets, and are an important part of platelet derived coagulation, as covered later (Fujii et al., 2015) . Platelet activation results in appearance of phosphotidylserine (PS) on the outer cell membrane which acts as a catalyst for procoagulant activity, along with the presence of tissue factor (TF), another coagulation trigger which further promotes the risk of thromboembolism. MP TF can be recycled by the endothelium and re-expressed on the endothelial surface (Collier et al., 2013). In healthy individuals, MP numbers are well managed, but have been shown to be increased, and associated with thromboembolism, in systemic vasculitis (Eleftheriou et al., 2011), childhood stroke (Eleftheriou et al., 2012), systemic sclerosis (Guiducci et al., 2008), rheumatoid arthritis (Norling and Dalli, 2013) and many other systemic inflammatory diseases (Roseblade et al., 2013).

There are three main processes required for MP release: rearrangement and breakdown of the regular cytoskeletal attachment to the plasma membrane, influx of calcium, and creation of the correct physical plasma membrane properties.

3.1.2 Cytoskeletal Rearrangement

3.1.2.1 *Rho family GTPases*

Rho family GTPases are a group of small G-proteins that have been shown to be involved in a large amount of actin rearrangement and regulation (Spiering and Hodgson, 2011). Rho-associated, coiled-coil-containing protein kinase 1 (ROCK-1) is critical for actin rearrangement processes involved in MP release (Kant et al., 2011). ROCK-1 is activated in most physiological situations by association with RhoA-GTP (Wójciak-Stothard et al., 1998).

RhoA is a Rho family GTPase associated with the plasma membrane. It is regulated by guanine nucleotide exchange factors (GEFs) which activate RhoA downstream function, and GTPase activating proteins (GAPs) which deactivate RhoA downstream function by increasing the rate of GTP hydrolysis. These GTP chaperones and GTPase activators are regulated by a range of upstream receptor families which include integrins, tyrosine kinase receptors, G-protein coupled receptors (GPCRs), cadherins, Ig receptors, and mechanical stresses. Additionally, there are Rho protein GDP dissociation inhibitors (Rho-GDI), which bind Rho-GDP, preventing Rho-GEF action and also dissociating Rho from the plasma membrane(Garcia-Mata et al., 2011). Rho-GDIs then enable regulation of the pool of RhoA that is functional at any one time (Figure 3.1).
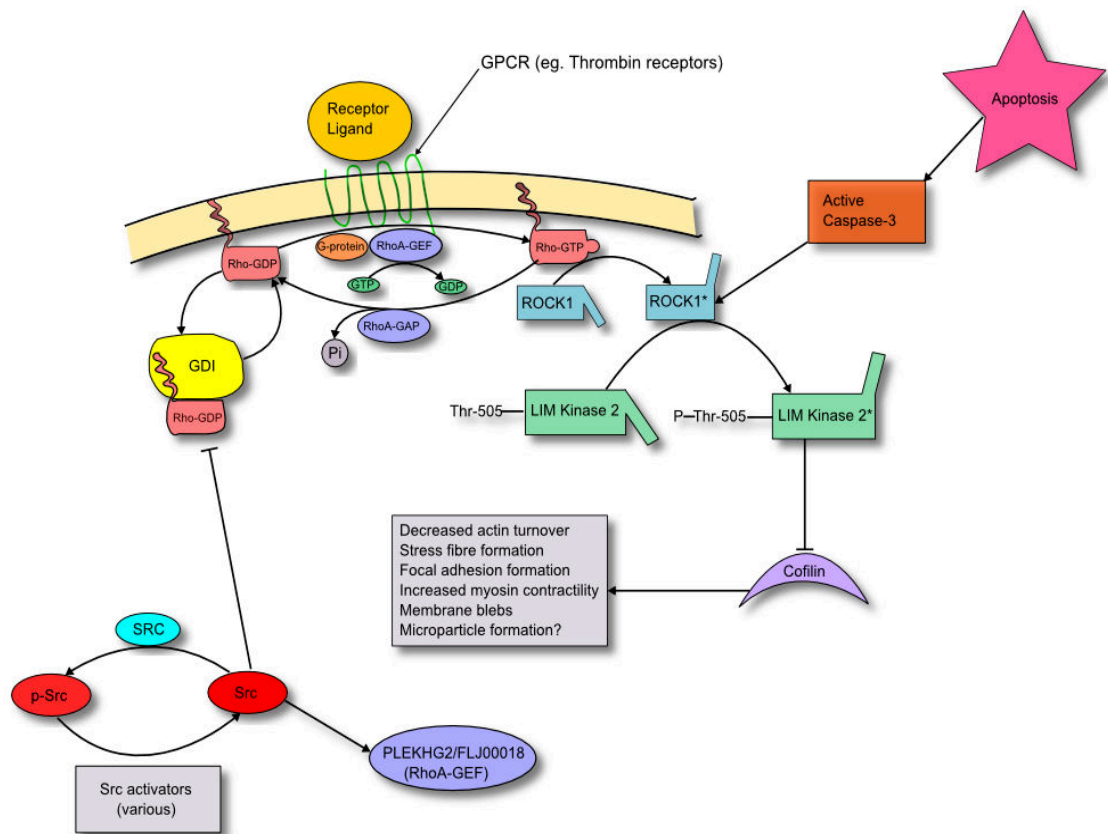
Figure 3.1: Rho/ROCK pathways involved in Microparticle release

When RhoA is in its active GTP-bound form, it is able to interact with ROCK-1; this interaction

leads to conformational changes in ROCK-1 with its C-terminal domain moving away from the

kinase domain. This kinase domain phosphorylates and activates LIM kinase 2 (LIMK2), which

in turn phosphorylates cofilin, inhibiting its actin-cleaving activity as highlighted in Figure 3.1

(Yang et al., 1998). This pathway reduces the turnover and creation of new F-actin, by reducing

the number of new "barbed" ends that actin polymerisation can occur from. This changes the

dynamic process of actin degradation and reconstruction, consequently affecting many cellular

processes including membrane blebbing, stress fibre and focal adhesion formation, and myosin

contractility (Amano et al., 2001).

Blebs are spherical outgrowths on the plasma membrane that occur when the cytoskeleton

becomes detached from the membrane, and occur in most cell types under a range of

conditions, including homeostasis. These blebs are thought to be inherent to MP formation.

LIMK2 has been shown to localise to the inside of partly-formed plasma membrane blebs, with an actin aggregate formed around the neck. Adding a ROCK-1 inhibitor to human umbilical vein endothelial cells (HUVECs) has been shown to prevent basal and activation-induced MP-production (Storck and Wojciak-Stothard, 2013). While not the full story, ROCK-1 inhibition has been shown to affect MP generation and looking at downstream proteins could point the way to other proteins involved.

ROCK-1 mediated signalling events are also an integral component of apoptosis. Once the apoptosis executor procaspase-3 has been activated, it cleaves off the C-terminal inhibitory domain of ROCK1, generating a constitutively active kinase that promotes cytoskeletal rearrangements, enabling the formation of apoptotic fragments. It has been shown that preventing MP release from endothelial cells causes their apoptosis and detachment, which is hypothesised to be due to lack of clearance in MPs of an accumulation of caspase-3 within the cell (Abid Hussein et al., 2007). These studies highlight the intimate crosstalk involved between apoptosis and MP formation.

3.1.2.2 *Src Tyrosine Kinase*

Another protein kinase implicated in MP release is Src, a non-receptor tyrosine kinase of the Src family. Src is a proto-oncogene that is overexpressed in ~50% of all tumours (Dehm and Bonham, 2004). Like RhoA, Src is activated in a variety of ways. In cellular homeostasis, the majority of Src is constitutively inactivated by the action of C-terminal Src kinase (CSK). This phosphorylates tyrosine residues on the C-terminal domain of Src and promotes conformational change into an inactive form. Src is then activated by either protein tyrosine phosphatases, or by physical displacement of its intramolecular interactions, exposing Tyr416 and allowing autophosphorylation, giving the active form of the kinase. This displacement and phosphorylation of Tyr416 can be achieved by receptors, such as epidermal growth factor receptor (EGFR). EGFR recruits a binding partner for Src, in this case p85-PI3K, which causes the conformational change and therefore activation. The displacement can also be achieved by

many other cytosolic proteins, and reflects the diverse triggers of Src activation (Liu et al., 2013). This explains the involvement of Src in a multitude of pathways.

Src is important as a potential regulator of MP formation as it can mediate the activation of Rho-family kinases, by phosphorylating GEFs. A variety of Rac/Cdc42-GEFs (such as Vav, Asef, from a related pathway) are tyrosine phosphorylated, which activates them and leads to activation of Rac/Cdc42. This has been shown in TNF-α stimulation of Rac/Cdc42 (Kant et al., 2011), and recently, for the first time, a Rho-GEF has been shown to be tyrosine phosphorylated by Src (Sato et al., 2014). The phosphorlyation of Rho-GDI proteins by Src leads to decreased interaction with Rho, increasing the pool of Rho at the plasma membrane (DerMardirossian et al., 2006). This increases the potential for Rho activation. Interestingly, Src also phosphorylates ezrin at Tyr477, regulating its activity (Mak et al., 2012). Ezrin is part of the ERM protein family that link the cytoskeleton to the plasma membrane, and this particular phosphorylation site has been shown to remove cell-cell contacts in epithelial cells, and reduce the invasiveness of a mouse mammary carcinoma cell line (Mak et al., 2012). Multifaceted functions of Src on the Rho-family members and other pathways result in a major impact on cytoskeletal remodelling. This effect however has not been studied in MPs.

Src has also been shown to bind to and phosphorylate caveolin-1 on Tyr 14, a membrane-associated protein that defines caveolae. These are small (50-100nm) invaginations of the plasma membrane that act as specialist lipid rafts (Figure 3.2) (Jiao et al., 2013). When phosphorylated, caveolin-1 opens store-operated calcium channels, such as transient receptor potential channel 1 (TRPC1), and increases cytosolic Ca^{2+}. This occurs via interaction of the caveolin scaffolding domain with the channels in a dose-dependent manner (Figure 3.2) (Rathor et al., 2014; Burger et al., 2011). This calcium increase activates calpains, cysteine proteases found within the cytosol. These then cause proteolytic cleavage of cytoskeletal proteins, such as talin and α-actin, causing membrane blebbing and MP formation (Pasquet et al., 1996). The influx of calcium itself also has a profound effect on the plasma membrane.
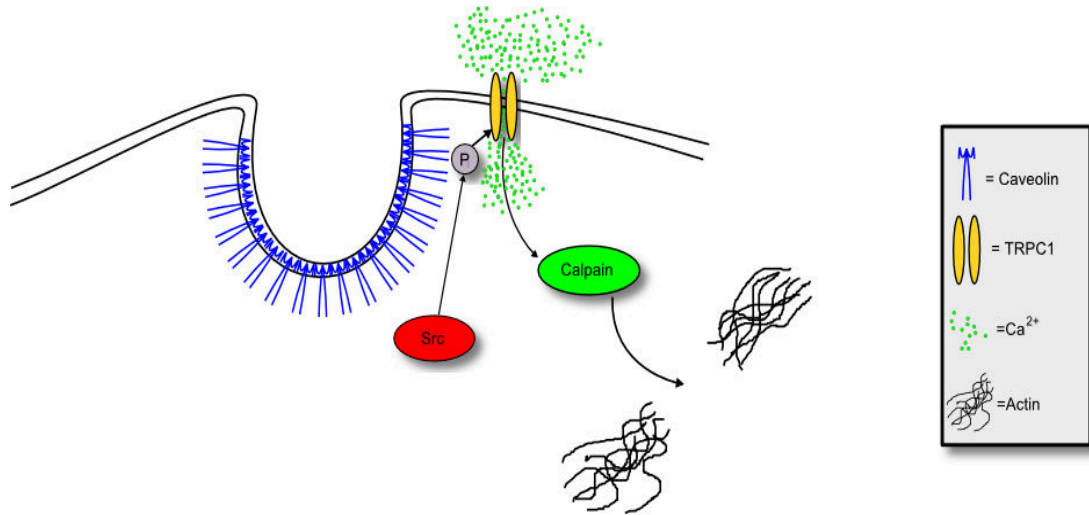
Figure 3.2: Active Src interacts with Caveolin, opening Ca^{2+} channels, activating Calpains and cleaving cytoskeletal actin and talin

3.1.3 Plasma Membrane Properties/Calcium Influx

3.1.3.1 Phosphatidylserine

To maintain homeostasis, the actions of two membrane translocation proteins keep an asymmetric lipid head group distribution across the inner and outer leaflets of the plasma membrane (Figure 3.3). Flippases translocate the negatively charged aminophospholipid phosphatidylserine (PS), and to a lesser extent the overall neutral phosphatidylethanolamine (PE), from the exoplasmic to the cytoplasmic leaflet of the plasma membrane in an ATP-dependent manner. Floppases, also in an ATP-dependent manner, translocate phosphatidylchoine (PC), sphingolipids, and cholesterol to the exoplasmic leaflet of the plasma membrane (Figure 3.3).
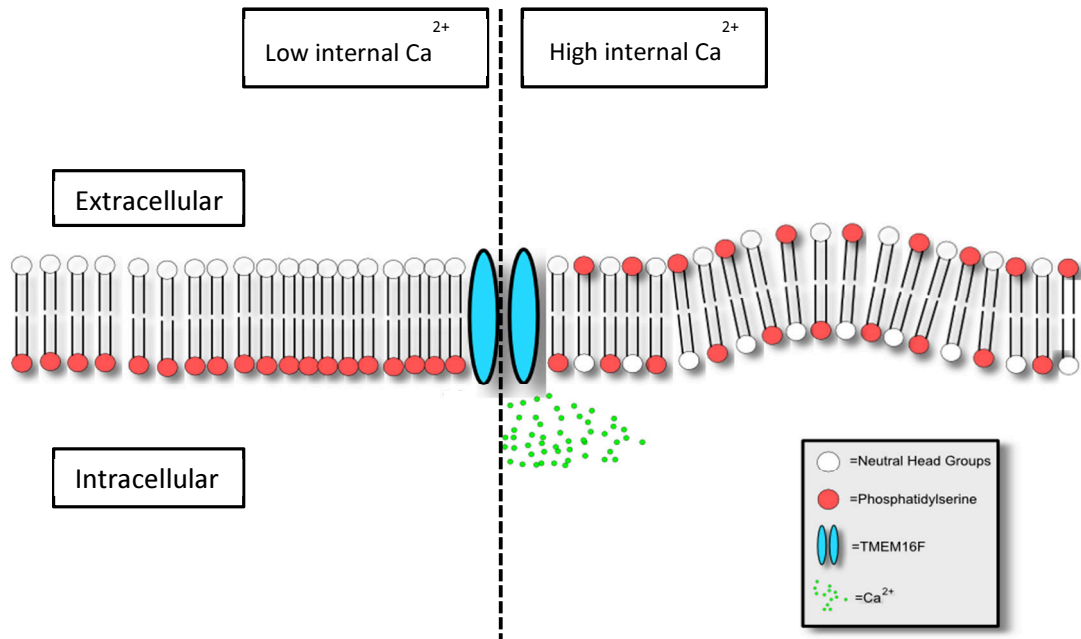
Figure 3.3: Calcium influx affects plasma membrane organisation and physical properties

The location of PS predominantly on the inner leaflet of the plasma membrane is known to influence the physical properties of the membrane, as addition of short-chain phospholipid analogues to the outer leaflet of platelet plasma membranes induce filopodia formation containing newly polymerised actin (Bettache et al., 2003). The restoration of the normal discoid platelet morphology only occurred if PS analogues were added to the outer leaflet, which were subsequently translocated to the inner leaflet via the flippase enzyme aminophospholipid transferase. This phenomenon shows that the altering of the lipid make up of each leaflet alone can have a major effect on the shape of the plasma membrane. PS on the inner leaflet of the plasma membrane has also been shown to organise lipid rafts, which recruit and allow co-localisation of Ras, Rho, Src, PKC family proteins and various receptors (Burger et al., 2011).

3.1.3.2   Calcium Influx

The asymmetric distribution of lipids across the leaflets of the plasma membrane is stable, with inactivation of flippases and floppases being insufficient to disrupt this arrangement (Bitbol et al., 1987). However the distribution is disrupted in a variety of physiological processes, including apoptosis, phagocytosis, platelet activation, and MP formation. PS is

exposed on the outer leaflet of the plasma membrane by scramblase activity, which is an ATP-independent lipid translocase (a protein that transports lipids across the plasma membrane along their concentration gradients, with relatively low specificity). The TMEM16 protein family members have been shown to have calcium-dependent scramblase activity with TMEM16F being specifically implicated in PS exposure, which fits the observations that PS exposure is calcium ion dependent (Suzuki et al., 2013). Introduction of a calcium ionophore that allows calcium to cross the plasma membrane and increase cytosolic Ca^{2+} is sufficient for PS exposure and MP release. This has been shown to be due to Ca^{2+} causing activation of scramblases, at the same time as inhibiting flippase activity (Suzuki et al., 2013; Bitbol et al., 1987).

It has also been shown that a K^+ efflux is required for MP release, with high extracelluar concentrations of K^+, or the addition of quinine, a calcium-activated K^+ channel blocker, preventing Ca^{2+} influx and therefore MP release (Campbell et al., 2014). Campbell et al have suggested that K^+ efflux is required to change osmotic pressure within the cell to allow reduction in cell volume, accounting for the loss of lipid surface area to MP. However, it has not been ruled out that K^+ efflux is not merely required to maintain a favourable electrochemical gradient so Ca^{2+} influx is not prevented by electrostatic repulsion of cytosolic K^+ ions. This hypothesis is supported by the work of Harper and Poole, who showed that preventing Cl^- influx via depleting Cl^- in the media, or inhibiting Cl^- channels, prevented PS exposure on platelets (Harper and Poole, 2013). Cl^- influx concurrently with Ca^{2+} could maintain membrane hyperpolarisation and allow sustained Ca^{2+} influx.

Ca^{2+} influx is physiologically very important for platelets, as PS exposure is required for platelet activation and initiation of the coagulation cascade, with PS acting as a catalytic surface for a variety of factors in the thrombin cascade. The importance of platelet activation is highlighted by the rare genetic condition Scott's Syndrome, where the mechanism of exposure of PS on activated platelets is defective, causing a hypocoagulable state and episodes of bleeding. In

the few cases of Scott's Syndrome that have been genetically characterised, mutations have been found in the TMEM16F protein, providing further evidence that this protein is responsible for Ca^{2+} dependent PS exposure (Castoldi et al., 2011; Suzuki et al., 2010).

3.1.3.3 Lipid Rafts

Lipid rafts are integral to the pathways involved in MP formation. Using two different lipid raft disrupters, namely methyl-β-cyclodextrin, a cholesterol solubiliser, and nystatin, which sequesters cholesterol, Burger et al showed disruption of lipid rafts prevented basal and angiotensin-II mediated MP release (Burger et al., 2011). While still a controversial concept, lipid rafts may provide a common platform for the molecular integration required for MP generation. The fact that lipid raft disruption prevents MP formation could be to do with preventing the correct interactions of Rho/GEFs/GAPs/ROCKs/Src/caveolin to facilitate MP release. It is interesting to note that proteomics studies of MP contents have discovered proteins known to associate with lipid rafts, although this is not conclusive proof of lipid raft involvement in their formation (Peterson et al., 2008).

Additional proteins of interest are Annexins, particularly Annexin A5 (AnV), which is known to reduce membrane permeability in the presence of calcium and reduce or reverse damage caused by a variety of agents (Creutz et al., 2012). This leads to the hypothesis that the calcium-dependence of the Annexin binding is a mechanism to specifically allow recruitment of Annexins to sites of damage or activation on the plasma membrane, as this is when cytosolic calcium increases. Combined with Annexin A5's specificity for PS, PE and PC, which all normally reside on the inner membrane leaflet until exposed by calcium-activated scramblase activity, this amounts to an elegant method of recruitment to damaged areas.

In summary, MP generation is a homeostatic phenomenon with a continuous, basal turnover. However, in many disease states there can be increased levels of MPs that leads to a hypercoagulable state (Owens and Mackman, 2011), but their role in inflammatory and autoimmune disease pathogenesis is an emerging area of research and remains ill-defined .

There have been few studies on MP production and thrombin generation in paediatric IBD (Deutschmann et al., 2013; Bernhard et al., 2011), and medical consensus is that further study and evaluation of these factors must be performed in order to recommend treatments, if any, to address the increased thromboembolic risk (Biss and Jaffray, 2014).

3.1.4   Statins

Statins are a group of small molecules that competitively inhibit the enzyme HMG-CoA reductase, and are primarily used as drugs to inhibit production of cholesterol, and thus treat hyperlipidaemia (National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), 2002). As larger datasets of longer-term use of statins has become available, they have been investigated for the treatment of many other diseases, such as improving outcomes after ischaemic stroke (O'Brien et al., 2015), treating multiple sclerosis (though no benefit was found) (Wang et al., 2011) and more generally reducing risk of cardiovascular disease in general populations (Taylor et al., 2013). It is important to note that long-term statin use reduced the circulating levels of endothelial, platelet and leukocyte derived MPs (Suades et al., 2013). However, an *in vitro* study of simvastatin on HUVECs demonstrated after acute exposure, there was an increase in detached endothelial cells and endothelial MPs (Diamant et al., 2008), which was ameliorated by the addition of the downstream product of HMG-CoA reductase, namely mevalonate. Increased endothelial MPs were also reduced with the addition of the further downstream molecule geranylgeranylpyrophosphate, suggesting that statins were acting on endothelial cells by reducing prenylation of other kinases, thus affecting their activity. The fact that these two results contradict each other highlights the pleiotropic effects of statins on cells and organisms, and that the range of their effects extends far beyond cholesterol lowering. The acute (<24h) effect of statins on MP release has not been studied, and may give an insight into the overall mechanisms by which statins change MP release.

3.2 Methods

3.2.1 Flow Cytometry

When analysing FACSCalibur flow cytometry plots of MPs, size was defined using side scatter rather than forward scatter due to the improved resolution seen at sub-micron sizes, as shown in general methods. MPs were defined first as <1µm in diameter when compared to 1µm diameter polystyrene beads (Figure 3.4A), and then positive for Annexin V (Figure 3.4B). Negative and positive gates for Annexin V were defined using MPs incubated with Annexin V in Ca^{2+}-free conditions, with PBS pH 7.4 replacing Annexin V buffer as a negative control (Figure 3.4B). Negative gates for antibodies specific to MP surface markers were defined using the appropriate IgG isotype control at the same 1 in 50 dilution as the antibodies used (Figure 3.4C).
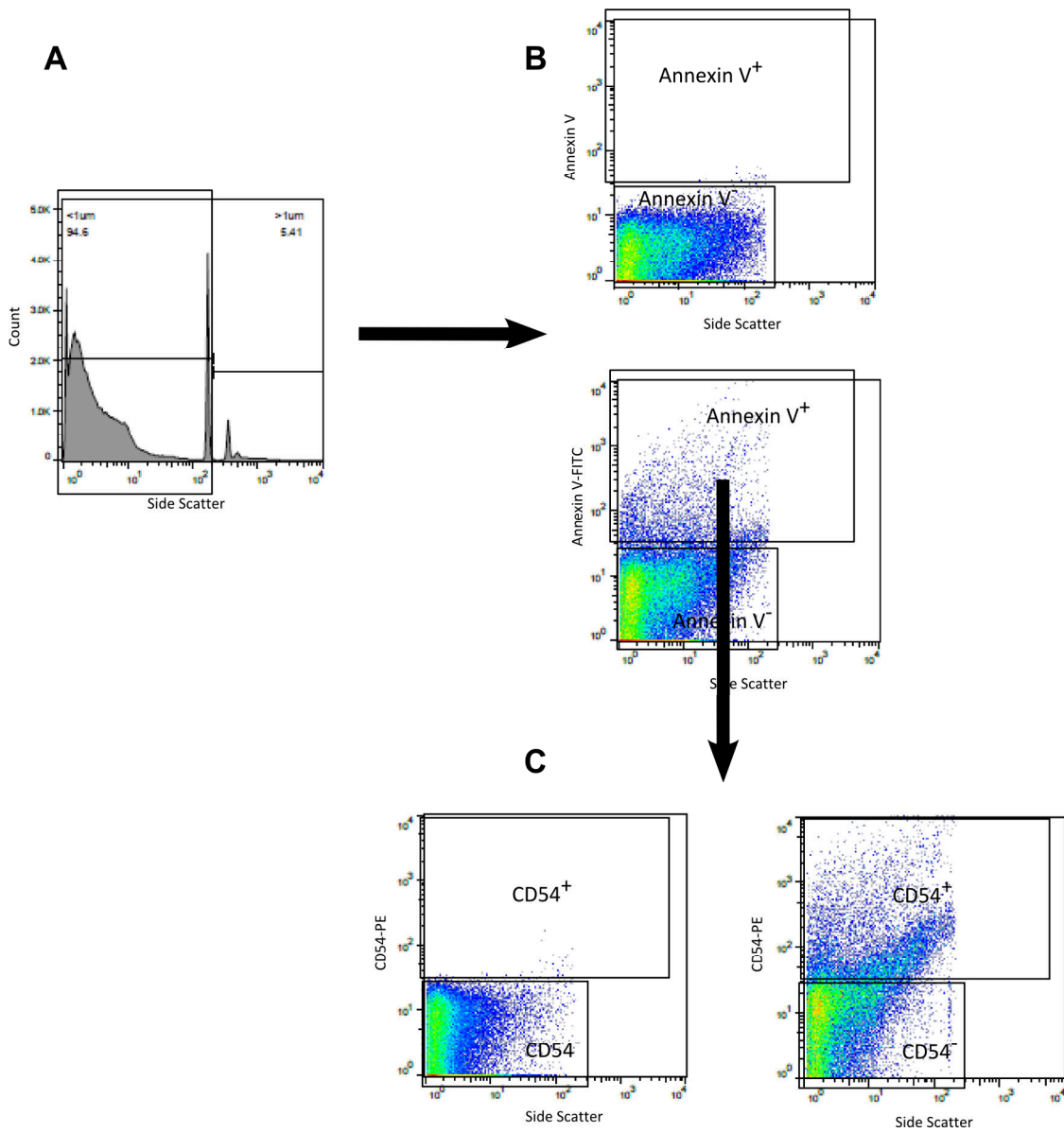
Figure 3.4: Gating strategy on FACSCalibur for Microparticle (MP) analysis

(A) 1µm polystyrene beads were run through the FACSCalibur flow cytometer to define particles <1µm in diameter by side scatter. (B) MP samples were stained with Annexin V-FITC, with positive and negative gates for this general MP marker defined by a negative control, namely, an MP sample incubated with Annexin V-FITC in Ca²⁺-free conditions. MPs were defined as <1µm, Annexin V positive. (C) These particles were stained and gated on their staining for surface markers, in this case CD54. Positive and negative gates were set using an isotype control.

3.2.2 Autologous Microparticle (MP) stimulation

EA.hy926 cells were grown in 6-well plates until fully confluent, and then stimulated with 50ng/mL TNF-α. Multiple instances of each condition were set up; when the resulting 1mL supernatant was harvested, one aliquot was assessed for IL-8 concentration by ELISA, and one aliquot assessed for MP enumeration by flow cytometry. A further 1mL aliquot was centrifuged at 20,000g for 1h at 4°C. The supernatant was carefully aspirated until 20µL was left in the tube, and the resultant pellet (usually invisible) was resuspended in 1mL PBS pH 7.4. This was centrifuged again at 20,000g for 1h at 4°C, and the supernatant carefully aspirated until 20µL was left in the tube. To investigate the potential inflammatory effect of the released MPs, the pellet containing released MPs was resuspended in 1mL DMEM media + 0.1% FCS and added to fresh, confluent EA.hy926 cells, and then incubated for 24h at 37°C, 5% CO_2. The supernatant was then aspirated and assessed for IL-8 concentration (detailed in Chapter 2.1.16) and MP enumeration.

3.3 Results

3.3.1 HUVEC and EA.hy926 cell-derived MP production

To determine the optimum FCS concentration to perform MP stimulation experiments at, as well as the ideal incubation time, EA.hy926 cells were stimulated with 50ng/mL TNF-α for 30min, 3h and 24h in 0-10% FCS (Figure 3.5). MPs produced were assessed as Annexin V (AnV) positive, and positive for the endothelial markers CD54 or CD62e. The only FCS concentration that showed a statistically significant increase in MPs using either surface marker was 0.1% FCS (Figure 3.5C,D). In all cases, any differences from control were only seen after 24h (AnV^{+}, CD54^{+} P<0.0001, AnV^{+}, CD62e^{+} P<0.001). Due to these results, further experiments conducted in EA.hy926 cells used DMEM + 0.1% FCS, and included the 24h incubation time.

For HUVECs, cells were stimulated with 10ng/mL TNF-α, IL-17A or interferon-γ (IFN-γ) for 30min, 3h or 24h, 0% or 10% FCS (Figure 3.6). While a statistically significant increase in AnV^{+}, CD54^{+} MPs was seen when stimulating with TNF-α in serum free conditions (Figure 3.6C, P<0.05), a greater increase was seen with 10% FCS (Figure 3.6**Error! Reference source not found.**D, P<0.05), and increases in TNF-α at 24h (P<0.05), IL-17A at 3h (P<0.0001) and IFN-γ at 3h (P<0.05) became significant in AnV^{+}, CD62e^{+} MPs when supplemented with 10% FCS. Due to these results, all further experiments were conducted in HUVECs were with 10% FCS supplementation.
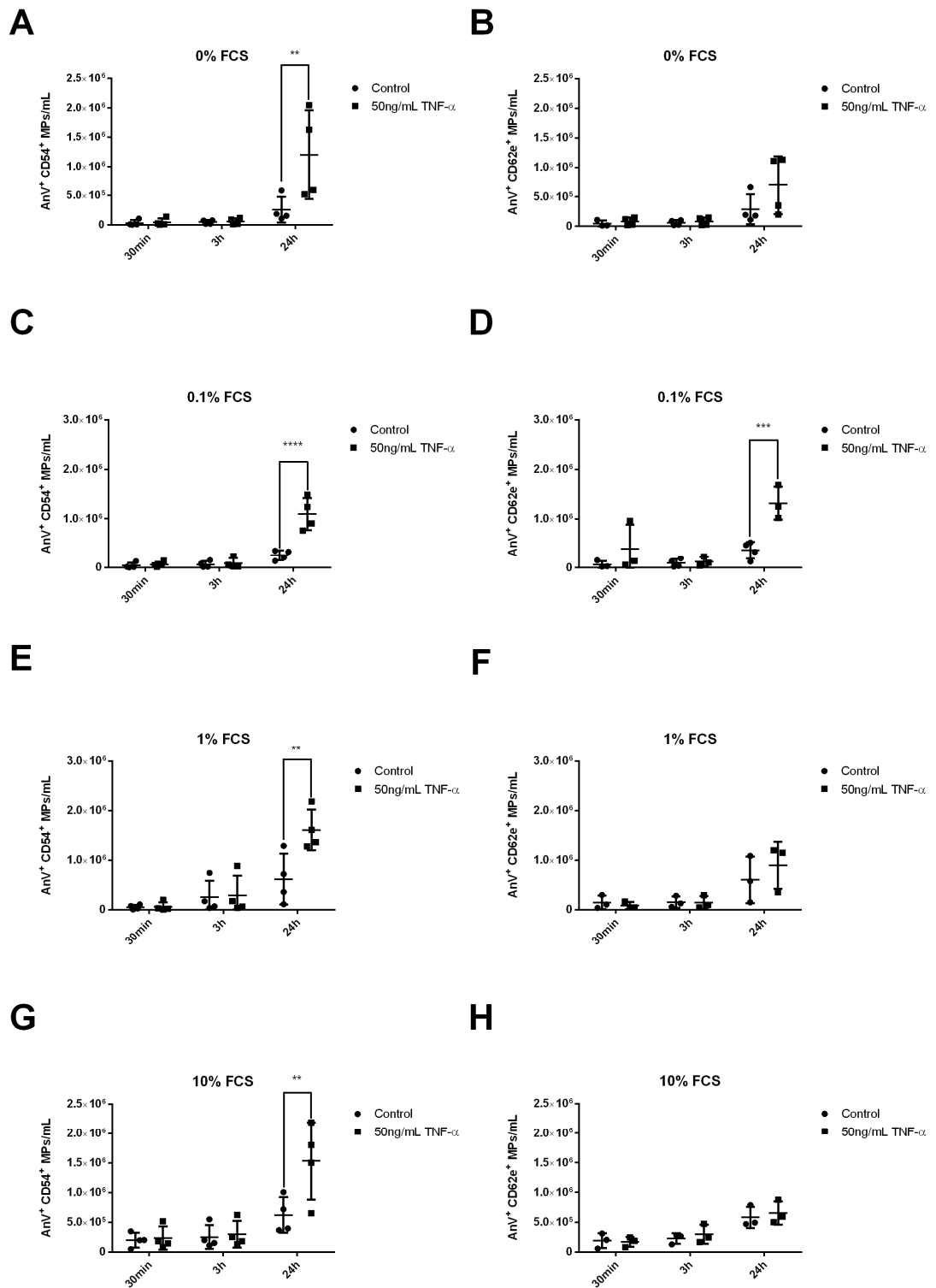
Figure 3.5: Impact of foetal calf serum (FCS) on TNF-α Stimulated Microparticle (MP) release from EA.hy926 cells.

Fully confluent EA.hy926 cells were incubated for 30 min, 3h or 24h in DMEM media supplemented with 0% **(A, B)**, 0.1% **(C,D)**, 1% **(E,F)** or 10% **(G,H)** FCS. All were stimulated with 50ng/mL TNF-α to stimulate MP release. Annexin V (AnV)$^+$, CD54$^+$ **(A,C,E,G)** and AnV$^+$, CD62e$^+$ **(B,D,F,H)** MPs were counted by flow

cytometry and compared to media-only controls. Results represented as mean and standard deviation,

with differences assessed by 2-way ANOVA with Sidak post-hoc multiple comparison. N=4, P<0.01 = **

P<0.001 = *** P<0.0001 = ****
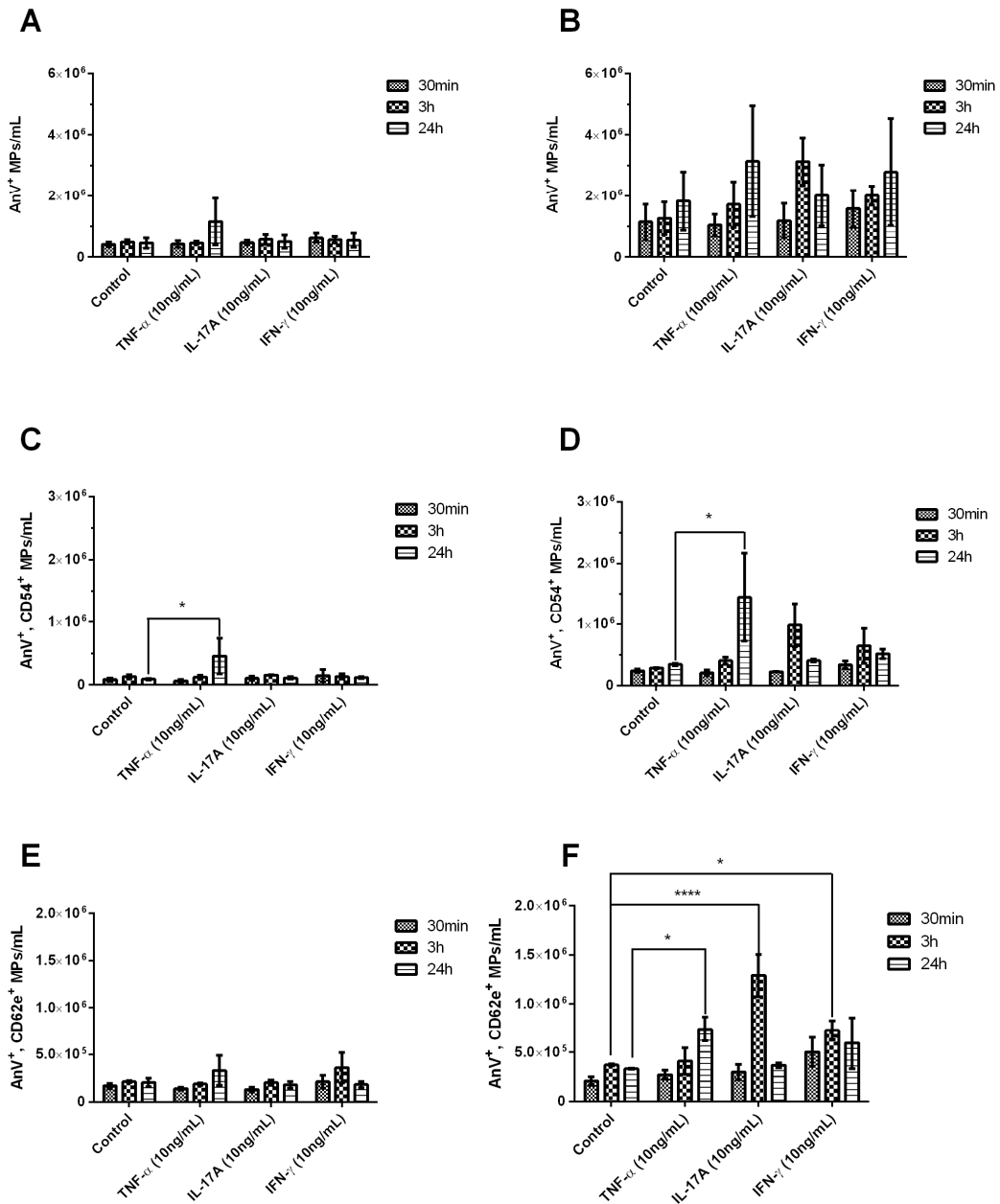
Figure 3.6: Impact of foetal calf serum (FCS) on TNF-α Stimulated Microparticle (MP) release from Human Umbilical Vein Endothelial cells (HUVECs).

*Fully confluent HUVECs in were incubated for 30 min, 3h or 24h in MCDB-131 media supplemented with 0% (A,C,E) or 10% (B,D,F) FCS. All were stimulated with 10ng/mL TNF-α, IL-17A or interferon-γ (IFN-γ) to stimulate MP release. Resulting Annexin V (AnV)$^+$ (A,B), AnV$^+$, CD54$^+$ (C,D) and AnV$^+$, CD62e$^+$ (E,F) MPs were counted by flow cytometry and compared to media-only controls. Results shown with mean and standard deviation, with differences assessed by 2-way ANOVA with Sidak post-hoc multiple comparison. N=4, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.*

3.3.2   Endothelial MP Stimulation by IBD cytokines

To ascertain if there is dose dependency or synergism of IBD-related cytokine stimulation of MP release from the endothelium, EA.hy926 cells were stimulated for 24h with 1-100ng/mL TNF-α, IL-17A, IFN-γ or 10ng/mL of all three cytokines. Stimulation with TNF-α showed an increase in AnV$^+$, CD54$^+$ (Figure 3.7A) and AnV$^+$, CD62e$^+$ (Figure 3.7B) MPs, regardless of the concentration used. There was no significant difference in the increase in either MP subset amongst concentrations of TNF-α tested. For both IL-17A and IFN-γ, none of the concentrations of cytokine used caused a significant increase in MP numbers of either subset. However, when combined as a three cytokine cocktail, there was a marked increase in both subsets of MPs (Figure 3.7), suggesting a synergistic effect of combining IBD-related cytokines on endothelial MP release.
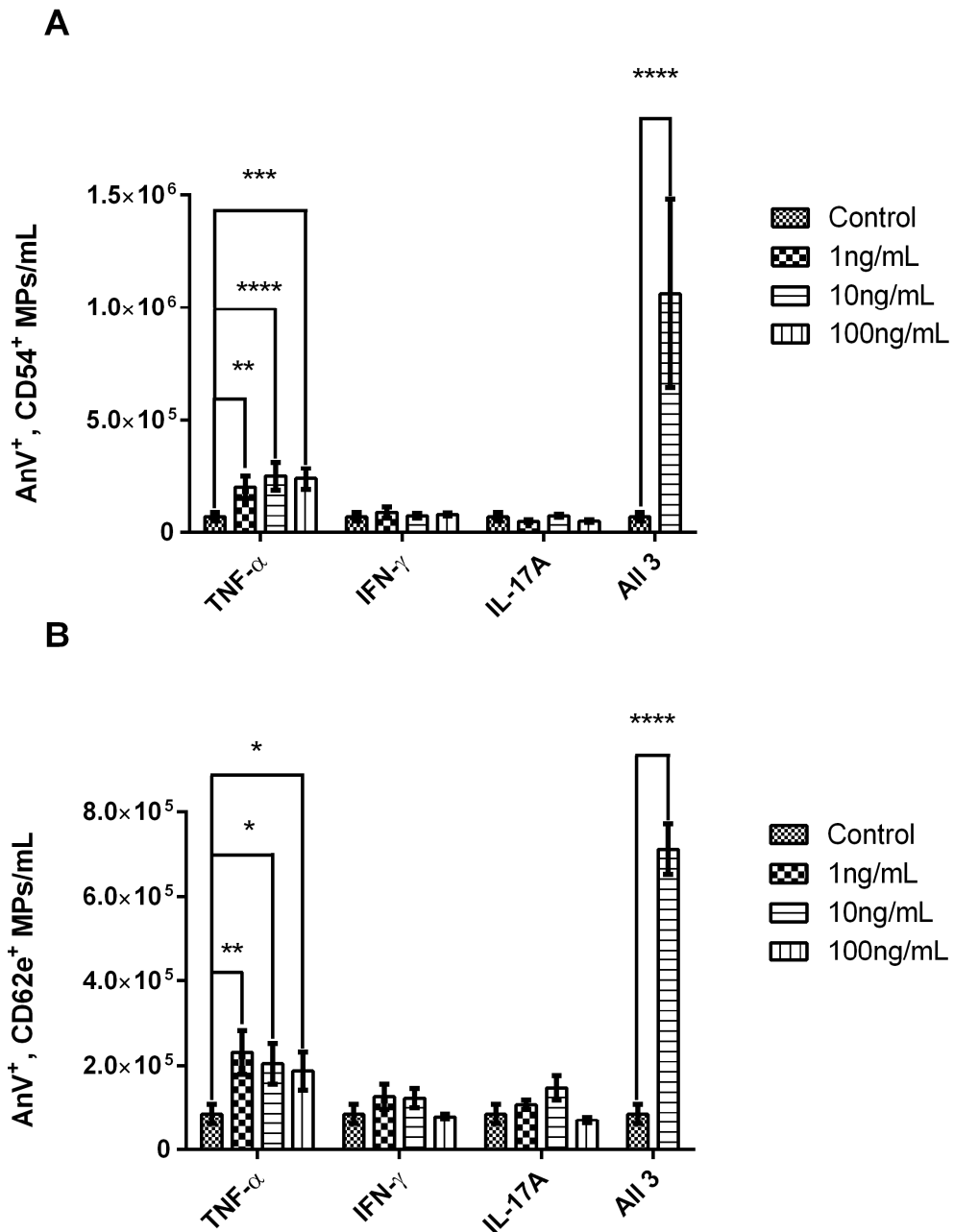
Figure 3.7: Dose dependent effect of IBD cytokines on EA.hy926 cell Microparticle (MP) release.

Fully confluent EA.hy926 cells were incubated for 24h in DMEM media supplemented with 0.1% FCS. All

were stimulated with 1ng/mL, 10ng/mL or 100ng/mL TNF-α, IL-17A, interferon-γ (IFN-γ), or 10ng/mL of

all 3 cytokines to provoke MP release. Resulting Annexin V (AnV)$^+$, CD54$^+$ (A) and AnV$^+$, CD62e$^+$ (B) MPs

were counted by flow cytometry and compared to media-only controls. Results shown with mean and

standard deviation, with differences assessed by 2-way ANOVA with Sidak post-hoc multiple comparison.

*N=4, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.*

3.3.3   Inhibition of Proposed MP release Pathways

EA.hy926 cells were incubated for 30min and 3h with 1µM of the Rho-associated kinase (ROCK) inhibitor GSK269962 (GSK), the LIM-domain kinase (LIMK) inhibitor LIMKi 3 or the Src inhibitor Src-1, and then assessed for MP production compared to controls containing 0.1% v/v DMSO (Figure 3.8). When looking at AnV$^+$ MPs (Figure 3.8A), after 30min there were no significant changes in MP numbers when incubated with any inhibitor. After 3h there was a trend for decreased AnV$^+$ MPs released when incubated with GSK, in contrast to a significant, ~2-fold increase in MPs released when incubated with LIMKi3. Src-1 incubation had minimal effect. The same trends and significant differences were seen in AnV$^+$, CD54$^+$ MPs (Figure 3.8B), with no changes in MP number after 30min and a statistically significant increase in MPs when incubated with LIMKi 3 for 3h. Conversely, when looking at AnV$^+$, CD62e$^+$ MPs (Figure 3.8C), there was a non-significant trend to decrease in MP numbers after 30min incubation with LIMKi 3. Additionally, after 3h there was a significant decrease in MPs when incubated with GSK. The increase seen in LIMKi 3-treated cells was not present when looking at AnV$^+$, CD62e$^+$ MPs.

When looking at the same inhibitors in HUVECs (Figure 3.9), there were no significant changes in AnV$^+$ (Figure 3.9A), AnV$^+$, CD54$^+$ (Figure 3.9B) or AnV$^+$, CD62e$^+$ (Figure 3.9C) MPs in any conditions tested. When incubated with GSK for 30min, there was a trend to decrease in MPs, especially AnV$^+$, CD54$^+$ (Figure 3.9B). Contrary to that seen in EA.hy926 cells, after 3h incubation there was no change in MPs released from LIMKi 3 or GSK treated cells, and Src-1 treated cells showed a large increase in MP number within all subsets. The high variability of this increase meant it was not statistically significant. Paradoxically, when incubating HUVECs with the respective inhibitors in addition to 10ng/mL TNF-α, IL-17A and IFN-γ for 3h (shown as 3h stimulated), the increase in MPs seen with Src-1 did not occur, and there were no significant increases in MPs with any inhibitor or within any subset of MPs.
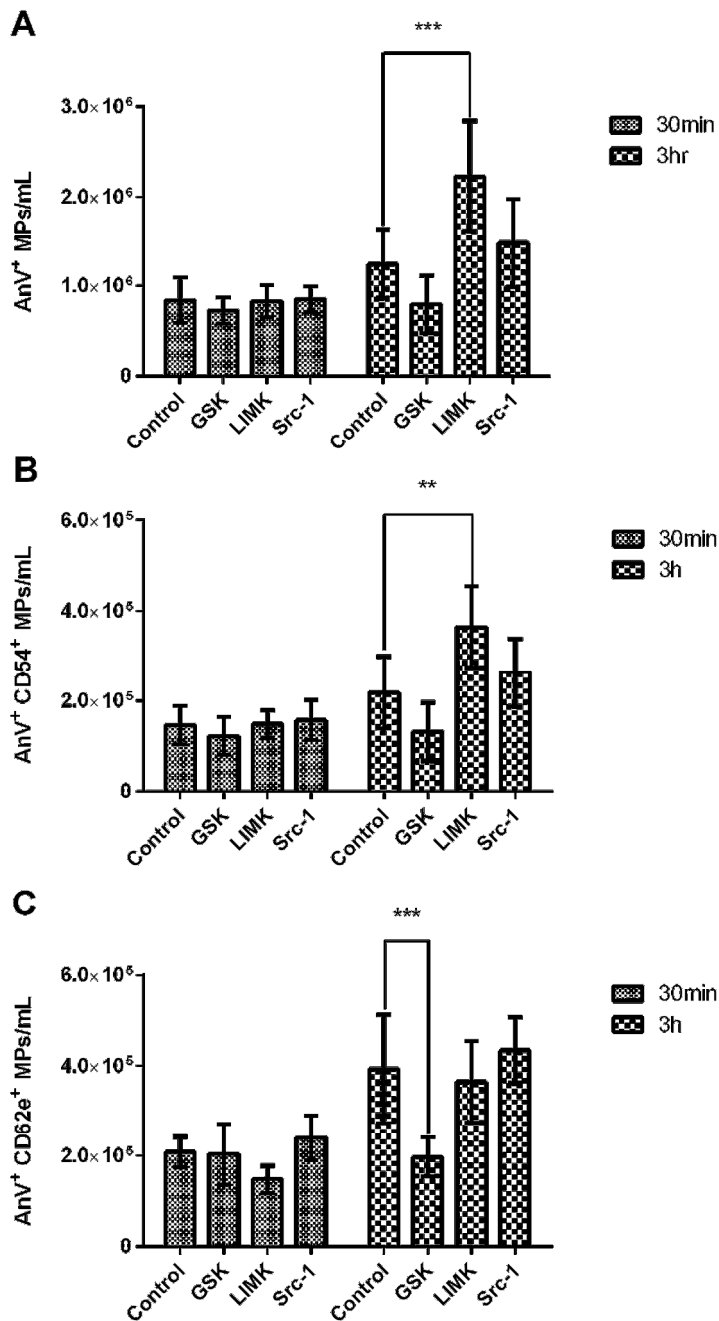
Figure 3.8: Inhibition of Rho-associated protein kinase (ROCK), LIM domain kinase (LIMK) and Src-1 by small molecules and their effect on microparticle (MP) release from EA.hy926 cells.

Fully confluent EA.hy926 cells were incubated for 24h in DMEM media supplemented with 0.1% FCS. Cells were also incubated with 1µM of the ROCK1 inhibitor GSK269962 (GSK), the LIMK inhibitor LIMKi 3 or the Src inhibitor Src-1. Annexin V (AnV)$^+$ **(A)**, (AnV)$^+$, CD54$^+$ **(B)** and AnV$^+$, CD62e$^+$ **(C)** MPs were counted by flow cytometry and compared to media + 0.1% dimethylsulphoxide (DMSO) controls. Results shown with

mean and standard deviation, with differences assessed by 2-way ANOVA with Sidak post-hoc multiple comparison. N=4, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.

A

B

C
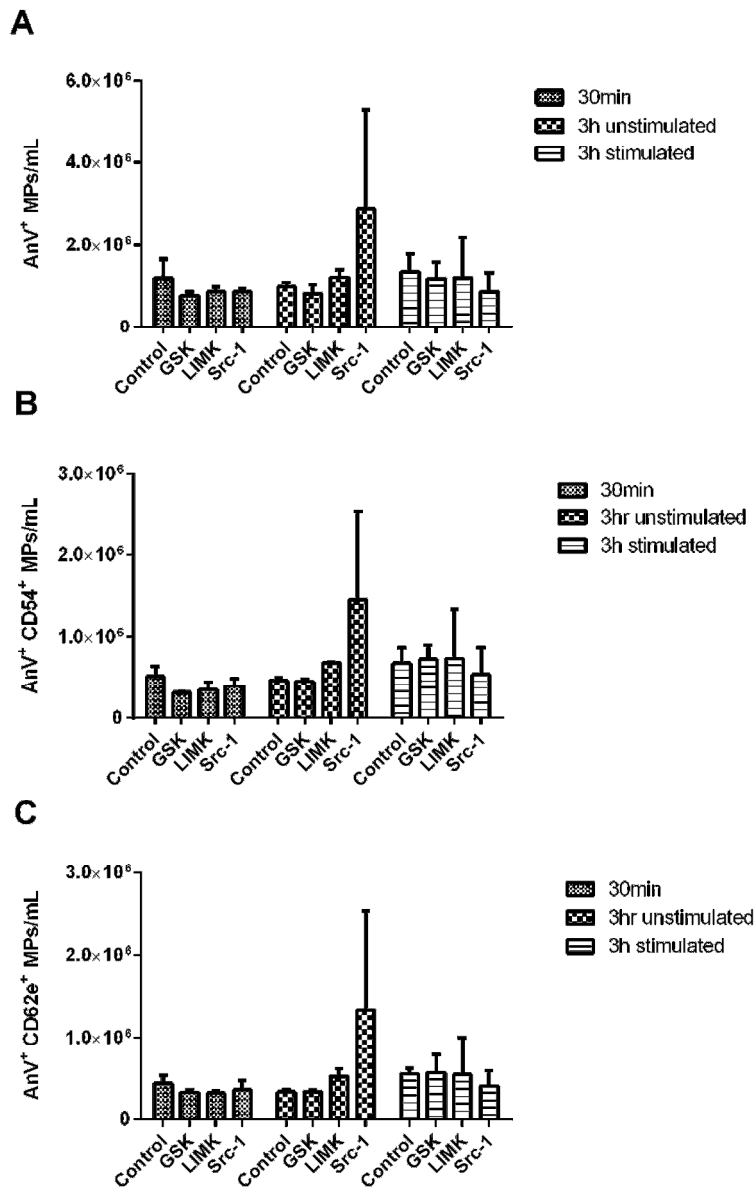
Figure 3.9: Effect of Inhibition of Rho-associated protein kinase (ROCK), LIM domain kinase (LIMK) and Src-1 on microparticle (MP) release from human umbilical vein endothelial cells (HUVECs .

*Fully confluent HUVECs were incubated for 24h in MCDB-131 media supplemented with 10% FCS. Cells were also incubated with 1µM of the ROCK1 inhibitor GSK269962 (GSK), the LIMK inhibitor LIMKi 3 or the Src inhibitor Src-1 for 30min, 3h or 3h with the addition of 10ng/mL TNF-α, IL-17A and IFN-γat the same time as addition of inhibitor. Resulting Annexin V (AnV)$^+$ **(A)**, (AnV)$^+$, CD54$^+$ **(B)** and AnV$^+$, CD62e$^+$ **(C)** MPs were counted by flow cytometry and compared to media + 0.1% dimethylsulphoxide (DMSO) controls. Results shown with mean and standard deviation, with differences assessed by 2-way ANOVA with Sidak post-hoc multiple comparison. N=4, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.*

3.3.4 Autologous MP stimulation

When EA.hy926 cells were stimulated with 50ng/mL TNF-α for 24h, a significant increase in AnV$^+$, CD54$^+$ MPs were released (Figure 3.10B). AnV$^+$, CD62e$^+$ MP production was increased, but not significantly (Figure 3.10C). In addition to MP release, the cells also showed significant increased production of IL-8 (Figure 3.10A). The increase in IL-8 was not unexpected as TNF-α is known to stimulate the endothelium and provoke downstream inflammation. We wished to explore if the subsequent MPs produced could exert an indirect effect on IL-8 production. When these MPs were isolated, washed and incubated with fresh EA.hy926 cells for 24h, MPs from TNF-α stimulated cells caused a significant increase in production of IL-8, compared to cells incubated with MPs from control conditions. This increase in IL-8 was not reflected in a corresponding increase in either subset of MPs (Figure 3.10B,C).
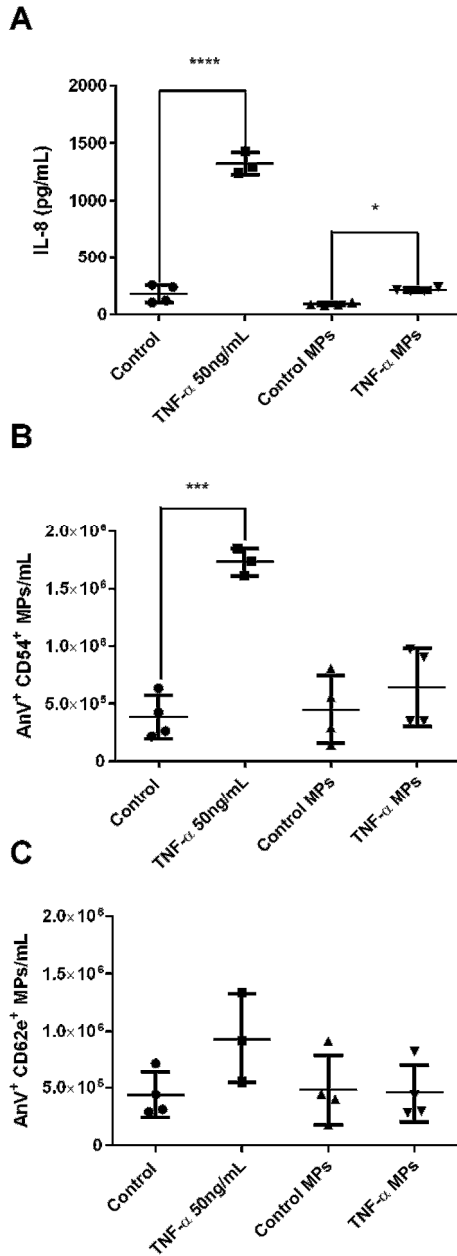
Figure 3.10: Autologous stimulation of EA.hy926 cells by TNF-α stimulated microparticles (MPs).

*Fully confluent EA.hy926 cells were incubated for 3h in DMEM media supplemented with 0.1% FCS, with the addition of 50ng/mL TNF-α, alongside a media-only control. MPs were harvested from the cell supernatants, washed in PBS pH 7.4 and added to fresh confluent EA.hy926 cells in media. Resultant production of IL-8 from each condition was assessed by ELISA **(A)**. (AnV)+, CD54+ **(B)** and AnV+, CD62e+ **(C)** MPs were counted by flow cytometry. Results shown with mean and standard deviation, with differences assessed by 1-way ANOVA with Tukey's post-hoc multiple comparison. N=3, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.*

3.3.5 Effect of Statins and Methotrexate on endothelial MP release

Addition of fluvastatin to EA.hy926 cells for 30min and 3h (Figure 3.11) showed no change in MP release compared to control in any subset of MPs investigated. This was confirmed with fluvastatin incubation with HUVECs (Figure 3.12), though with potentially additional variability between replicates. Addition of simvastatin to EA.hy926 cells for 30min and 3h (Figure 3.13) showed no change in MP release compared to control in AnV^+ (Figure 3.13A) and AnV^+, $CD62e^+$ (Figure 3.13C) MPs, but after 30min there was a significant decrease in AnV^+, $CD54^+$ (Figure 3.13B) MPs, which was not present after 3h incubation. Simvastatin incubation for 30min and 3h with HUVECs (Figure 3.14) showed no change in MP release compared to control in all subsets of MPs analysed.

Addition of the dihydrofolate reductase inhibitor methotrexate to EA.hy926 cells for 30min and 3h (Figure 3.15) showed no change in MP release compared to control in any subset of MPs investigated, and this was reflected and repeated in methotrexate incubation with HUVECs (Figure 3.16).
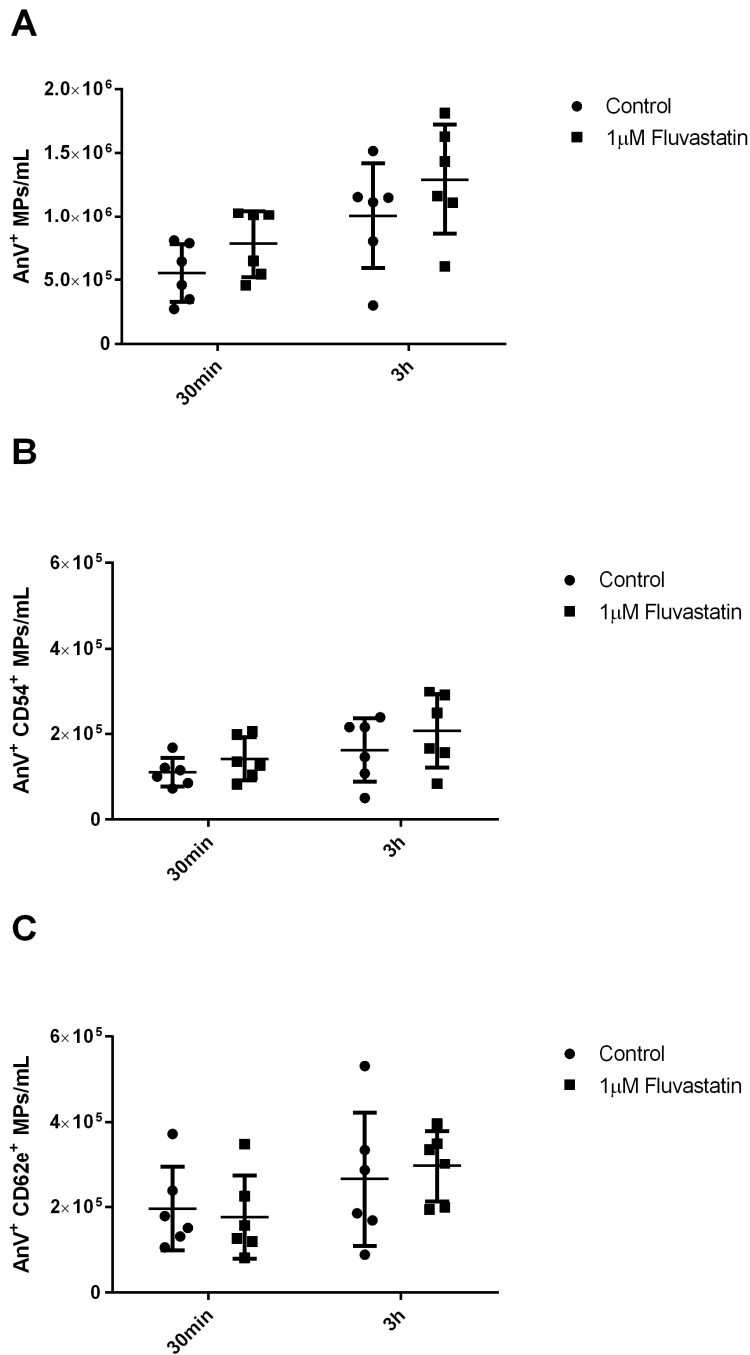
Figure 3.11: Effect of Fluvastatin on microparticle (MP) release from EA.hy926 cells.

Fully confluent EA.hy926 cells were incubated for 30min and 3h in DMEM media supplemented with 0.1% FCS, with the addition of 1μM Fluvastatin. Resulting Annexin V (AnV)$^+$ *(A)*, (AnV)$^+$, CD54$^+$ *(B)* and AnV$^+$, CD62e$^+$ *(C)* MPs were counted by flow cytometry and compared to media + 0.1% dimethylsulphoxide (DMSO) controls. Results shown with mean and standard deviation, with differences assessed by 2-way ANOVA with Sidak post-hoc multiple comparison. N=6, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.

A

B

C
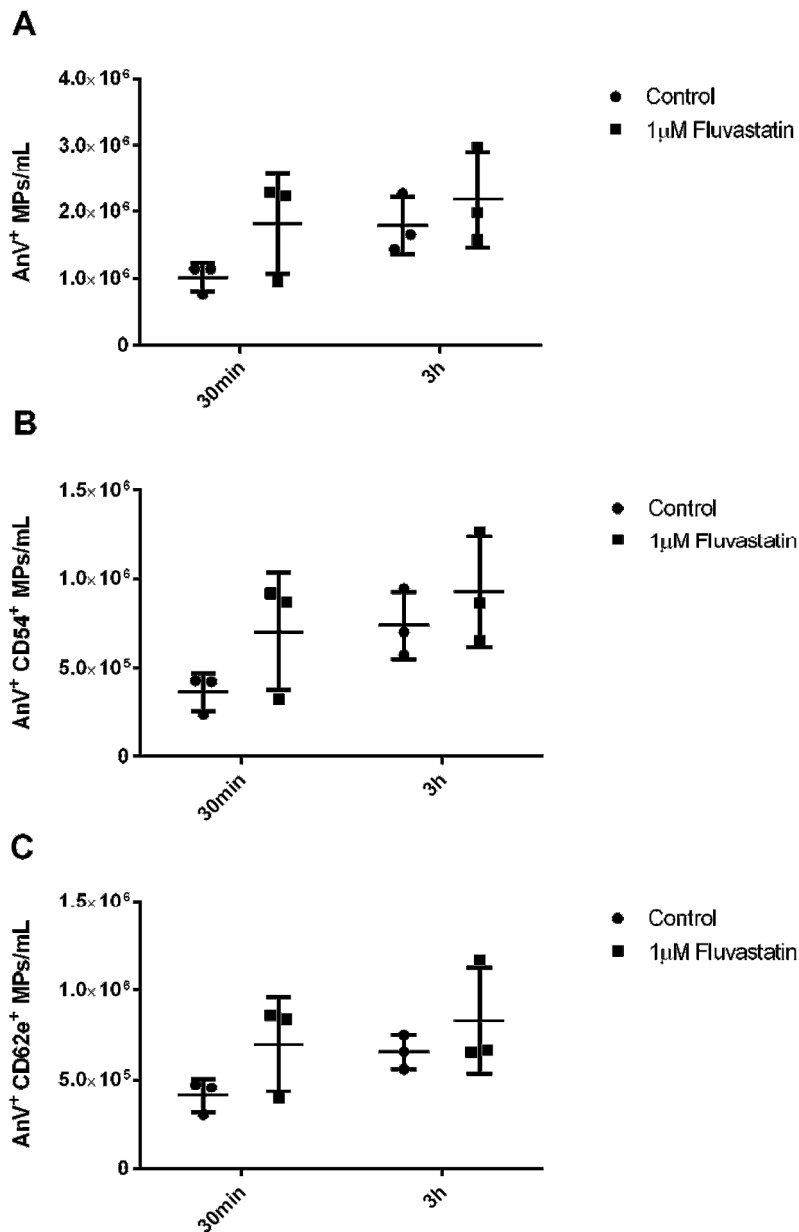
Figure 3.12: Effect Fluvastatin on microparticle (MP) release from human umbilical vein endothelial cells (HUVECs).

*Fully confluent HUVECs were incubated for 30min and 3h in MCDB-131 media supplemented with 10% FCS, with the addition of 1μM Fluvastatin. Resulting Annexin V (AnV)$^+$ (A), (AnV)$^+$, CD54$^+$ (B) and AnV$^+$, CD62e$^+$ (C) MPs were counted by flow cytometry and compared to media + 0.1% dimethylsulphoxide (DMSO) controls. Results shown with mean and standard deviation, with differences assessed by 2-way ANOVA with Sidak post-hoc multiple comparison. N=3, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.*
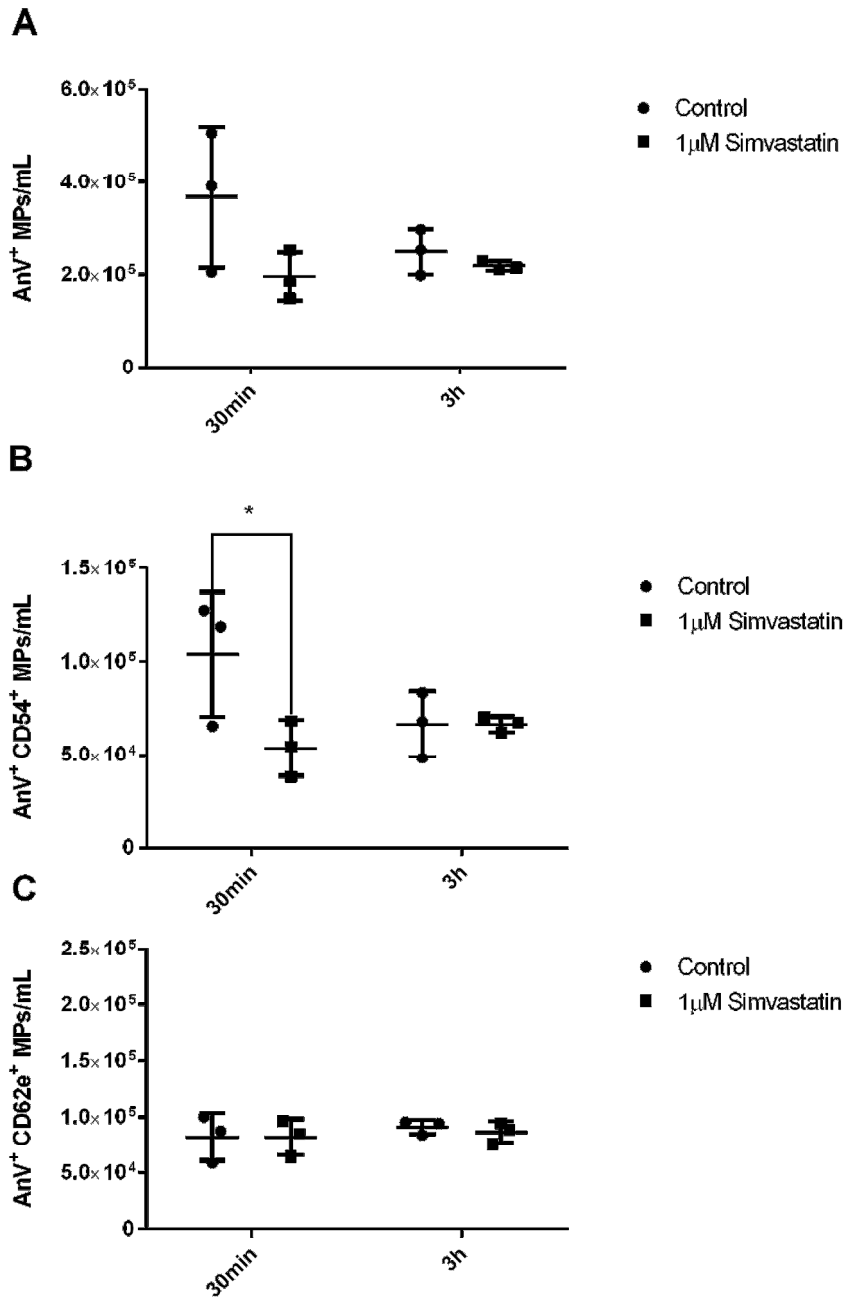
Figure 3.13: Effect of Simvastatin on microparticle (MP) release from EA.hy926 cells.

*Fully confluent EA.hy926 cells were incubated for 30min and 3h in DMEM media supplemented with 0.1% FCS, with the addition of 1μM Simvastatin. Resulting Annexin V (AnV)$^+$ (A), (AnV)$^+$, CD54$^+$ (B) and AnV$^+$, CD62e$^+$ (C) MPs were counted by flow cytometry and compared to media + 0.1% dimethylsulphoxide (DMSO) controls. Results shown with mean and standard deviation, with differences assessed by 2-way ANOVA with Sidak post-hoc multiple comparison. N=3, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.*
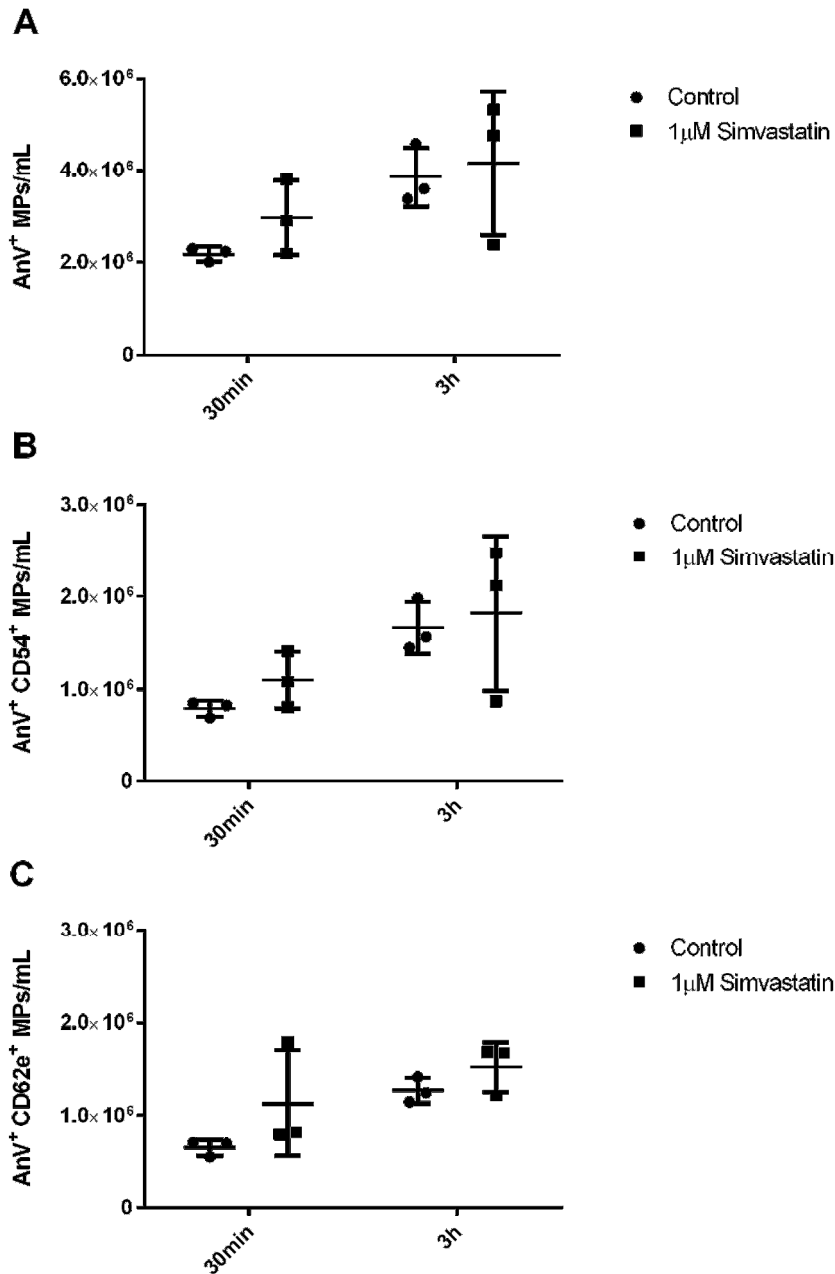
Figure 3.14: Effect of Simvastatin on microparticle (MP) release from human umbilical vein endothelial cells (HUVECs).

*Fully confluent HUVECs were incubated for 30min and 3h in MCDB-131 media supplemented with 10% FCS, with the addition of 1μM Simvastatin. Resulting Annexin V (AnV)$^+$ **(A)**, (AnV)$^+$, CD54$^+$ **(B)** and AnV$^+$, CD62e$^+$ **(C)** MPs were counted by flow cytometry and compared to media + 0.1% dimethylsulphoxide (DMSO) controls. Results shown with mean and standard deviation, with differences assessed by 2-way ANOVA with Sidak post-hoc multiple comparison. N=3, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.*

A

B

C
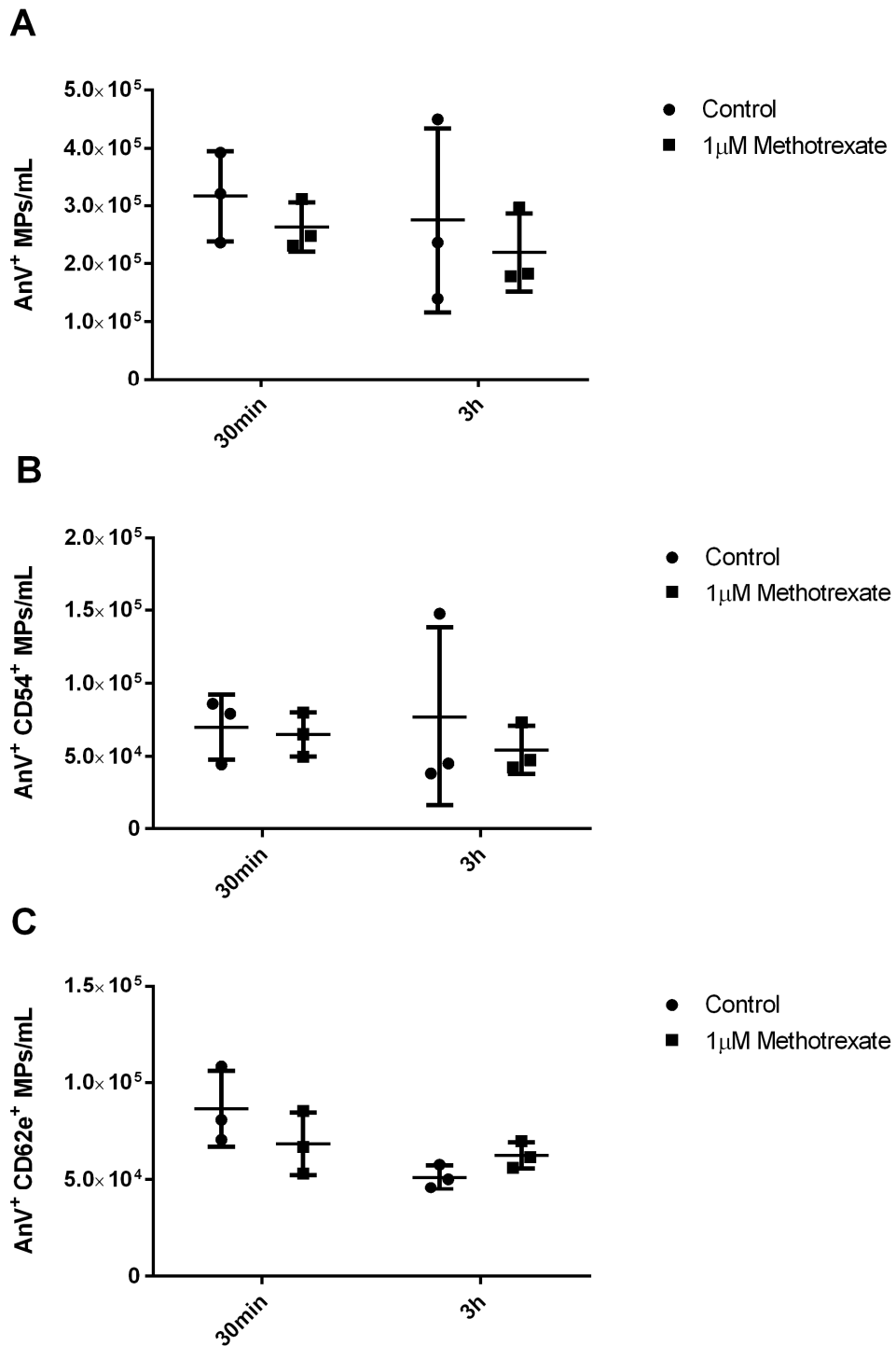
Figure 3.15: Effect of Methotrexate on microparticle (MP) release from EA.hy926 cells.

Fully confluent EA.hy926 cells were incubated for 30min and 3h in DMEM media supplemented with

*0.1% FCS, with the addition of 1μM Methotrextate. Resulting Annexin V (AnV)$^+$ **(A)**, (AnV)$^+$, CD54$^+$ **(B)** and*

*AnV$^+$, CD62e$^+$ **(C)** MPs were counted by flow cytometry and compared to media controls. Results shown*

with mean and standard deviation, with differences assessed by 2-way ANOVA with Sidak post-hoc

*multiple comparison. N=3, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.*

A

B

C

Figure 3.16: Effect of Methotrexate on microparticle (MP) release from human umbilical vein endothelial cells (HUVECs).

*Fully confluent HUVECs were incubated for 30min and 3h in MCDB-131 media supplemented with 10% FCS, with the addition of 1μM Methotrexate. Resulting Annexin V (AnV)$^+$ (A), (AnV)$^+$, CD54$^+$ (B) and AnV$^+$, CD62e$^+$ (C) MPs were counted by flow cytometry and compared to media controls. Results shown with mean and standard deviation, with differences assessed by 2-way ANOVA with Sidak post-hoc multiple comparison. N=3, P<0.05 = * P<0.01 = ** P<0.001 = *** P<0.0001 = ****.*

3.4 Discussion

The precise mechanisms involved in endothelial MP release in homeostasis and their contribution to disease pathogenesis currently remain unclear. In the present study, an exploratory *in-vitro* investigation was conducted to identify optimal conditions for studying endothelial MP release. In initial experiments, MP release from a high passage number HUVEC/sarcoma hybrid endothelial cell-line (EA.hy926) were compared with responses from freshly isolated HUVECs from umbilical cords. It was interesting to record a significant difference in the requirement for FCS and MP release between the two cell types. When incubated with 10% FCS, control levels of MPs released from HUVECs were consistently higher than in serum-free conditions (Figure 3.6), but this was more than offset by the far greater response to stimulation with addition of FCS. This would suggest that growth factors, hormones and proteins found in FCS are required to maintain the metabolic processes of HUVECs, so that they can respond to cytokine stimulation. This is a valid suggestion, as the natural physiological environment for the vascular endothelium is to be bathed in near 100% serum, i.e. blood. However, it is normally good practice in cell culture to minimise the serum supplementation required during experiments in order to reduce the effects of growth mediators present in FCS on the experimental system. It is also good practice to perform all experiments with the same batch of FCS, as levels of proteins and growth factors can vary from batch to batch, affecting the base level of MP release. When looking at the ideal FCS concentration for EA.hy926 MP experiments, the optimal concentration was substantially lower, at 0.1% (Figure 3.5). This likely reflects the fact EA.hy926 cells are a cancer hybridoma and do not require the pro-survival growth factors due to constitutively active pro-survival signals, for example Src. This highlights the trade off, that while EA.hy926 cells can be used in a practically serum-free media, they have vastly altered metabolism compared to primary cells.

An important drawback to the *in vitro* vascular endothelium systems that have been used is the lack of sheer stress applied to the cells. *In vivo*, the vascular endothelium is subject to

laminar flow of blood, which gives a constant sheer stress to the surface of cells. When replicated *in vitro*, this laminar sheer stress reduces overall MP production (J.-S. Kim et al., 2015) in a mitochondrial dependent manner. The fact there was no provision to subject cells to laminar flow during incubation means all cells produced a higher basal level MPs, and future work must consider investigating endothelial MP release under laminar flow conditions, or using an *in vivo* model. It may be that with the removal of this physiological stress, endothelial cells may respond more acutely to cytokine stimulation. The lack of laminar flow may also account for the lack of MP inhibition seen with most inhibitors used, even though the pathways targeted have been shown to be involved in MP generation.

The cytokines chosen to stimulate MP release (TNF-α, IL-17A, IFN-γ) were selected both for their known ability to stimulate MP release (Jimenez et al., 2003), but additionally for their relevance to the pathogenesis of IBD. TNF-α is a well-studied cytokine shown to be heavily involved in IBD pathogenesis; the anti-TNF-α biologics infliximab and adalimumab are used as a powerful treatment for severe IBD (Van Assche et al., 2011; Roblin et al., 2014). IL-17A is the prototypical cytokine released from Th17 cells, and has been shown in mouse models to be associated with IBD (Cătană et al., 2015), as well as synergistically acting with TNF-α to promote endothelial MP release (Hot et al., 2012). IFN-γ has been implicated in IBD pathogenesis (Rafa et al., 2010) but its role hasn't been entirely discerned. The data presented show that while each cytokine individually can increase endothelial MP production, the synergistic effect of adding all three cytokines exerts a more potent effect (Figure 3.7). This is important, as it is far more likely that patients with IBD will have multiple raised cytokines at once, and shows the effects of these cytokines should always be considered as a collective as they can reinforce the effects of each other. Future experiments could explore this by comparing the effect of IBD patient plasma on endothelial MP release compared to healthy plasma.

Another important finding from these experiments is that the effect that MPs have on further cells is dependent on the conditions in which they were produced, i.e. MPs are not created equal. When harvested from TNF-α stimulated cells, MPs were able to stimulate IL-8 release from fresh vascular endothelial cells (Figure 3.10). This shows the potential for MPs to possess pro-inflammatory properties, and may therefore act as a vehicle for continuing inflammation systemically from a local source. Pro-inflammatory MPs could help to propagate inflammation throughout the body, and could theoretically contribute to IBD pathogenesis. Reducing MP production is an interesting avenue of enquiry for helping to reduce inflammation.

Statins have been shown to reduce circulating endothelial MPs *in vivo* (Suades et al., 2013), and methotrexate is an anti-folate drug commonly used in the treatment in IBD, with a potential for also reducing circulating MPs (Pelletier et al., 2014). The ineffectiveness of fluvastatin and methotrexate at modifying MP release acutely in these endothelial systems (Figure 3.11Figure *3.12*, Figure 3.15Figure *3.16*) may either point to a lack of effect in the short term, or it could be that the model system is not sensitive enough to detect any changes. There was a reduction in AnV⁺, CD54⁺ MP release after 30min treatment with simvastatin in EA.hy926 cells, but this reduction disappeared after 3h (Figure 3.13). The previous comment on laminar flow and its effect on basal MP release could be a deciding factor in this case. It may be that in an artificial *in vitro* system the endothelium is not sensitive enough to these treatments, and would be better investigated in a more physiological *in vivo* murine model. This same argument could be made for the small molecule inhibitors. An interesting point to be made about Src inhibition is that the large (but insignificant due to variability) increases in MPs seen after 3h Src inhibition in HUVECs (Figure 3.9) were ameliorated with the addition of cytokine, and are not present at all in EA.hy926 cells (Figure 3.8). The fact that EA.hy926 cells are a cancer line and Src is constitutively overexpressed in 50% of all tumours (Dehm and Bonham, 2004) means that a higher Src-1 concentration would be needed to inhibit Src activity in EA.hy926 cells. The fact that the increase in MPs was reduced with addition of pro-inflammatory cytokines in HUVECs and that Src is pro-proliferative, suggests after 3h of

inhibition HUVECs could be showing increased levels of apoptosis, which would explain the observed increased MPs. When pro-inflammatory cytokines are added the subsequent NF-κB activation could provide an alternative pro-proliferation signal and prevent Src inhibition-induced apoptosis. A potential way to show this would be to look at the percentage of viable cells using trypan blue dye under each condition, to determine levels of cell death.

4. Vascular Complications and Thromboembolic Risk in Paediatric IBD: Clinical Study Recruitment

4.1 Introduction

In order to address the aims and hypotheses put forward at the end of Chapter 1, a comparative clinical study was conducted. In the present study, we tested the hypothesis that even in well-managed inactive or mild IBD, ongoing chronic subclinical inflammation promotes increased cardiovascular risk by causing vascular endothelial dysfunction. This results in an increase in circulating MPs (a newly established systemic inflammatory mediator (Collier et al., 2013; Eleftheriou et al., 2011)), thrombin generation potentially causing a pro-coagulation state, and increased arterial stiffness. This is the first study detailing multiple parameters in a single cohort, providing a more comprehensive analysis of cardiovascular risk in paediatric IBD. The study design, recruitment and patient demographics are detailed below.

4.2 Methods

4.2.1 Study Design and Ethical Approval

This was a comparative, observational study with full ethical approval, organised, written and defended at ethics committee by myself (14/LO/0644). Participants >16-years of age provided fully-informed written consent; written parental consent was obtained for those <16-years, with additional written assent obtained from the young participant. Paediatric IBD outpatients were recruited at University College London Great Ormond Street Hospital (UCL, GOSH), between March 2014 and March 2016. Healthy adolescent controls were recruited as part of science research study days [Rayne Institute, UCL; January-March 2016, ethics (08/H0713/80)]. To obtain age and sex-matched control data for investigation of arterial stiffness, data from 34 participants from a previous study into effects on the vasculature from Kawasaki Disease, using the same methodology by our group were used (Shah et al., 2015). Healthy controls were

recruited for this historical study from unaffected siblings of Kawasaki Disease patients treated at GOSH. De-identified clinical data collated included age, sex, age at diagnosis, current treatments, BMI, blood pressure, smoking status and family history of death by cardiovascular disease. All data relating to participants was pseudoanonymised, with an unrelated identifying number assigned to each record. There was a single hard-copy code break stored in a locked filing cabinet, in a locked office, to allow identification and removal of participant records if a participant decided to withdraw from the study.

For IBD patients, inclusion criteria were age between 7.5 and 18.5 years at time of study, and a clinical diagnosis of IBD (either CD, UC or IBD-U) confirmed by endoscopy. Disease activity was assessed at the time of recruitment by the ImproveCareNow (Crandall et al., 2011) Physicians Global Assessment (PGA) score, with patients categorised as inactive (quiescent) or active (only mildly active patients were recruited). Inclusion criteria for controls were also age 7.5 to 18.5 years at time of study, with exclusion criteria for both groups being any significant acute or chronic intercurrent illness or process, such as an infection, or history of cardiovascular disease in parents or grandparents.

4.2.2   Sample Collection

All IBD blood was obtained concurrently with other blood tests as part of participants' routine care. Adolescent control blood was obtained simultaneously with other research studies, with consent. A tourniquet was used around the upper arm before venepuncture with a 21-gauge needle from the antecubital vein; the first 5mL of blood drawn was excluded from our study to prevent endothelial cell contamination from the puncture site. Samples were collected in tubes containing sodium tricitrate or EDTA as appropriate. All samples were centrifuged at 5000 x g for 5mins, with plasma aspirated and re-spun under the same conditions, except those allotted for circulating endothelial cell (CEC) enumeration, which were left as whole blood. The platelet poor plasma (PPP) samples were stored at -80°C until required.

4.3 Results

4.3.1 Participants Recruited

In total 35 paediatric IBD patients were recruited (median age 13.7y (7.8-16.5), 63% males, median BMI 21.8 (16.2, 27.0); Table 1): 20 CD, 8 UC and 7 IBD-U (Table 4.1). All were classified by PGA score as inactive (quiescent, n=27) or active (mild, n=8) IBD. Current treatments are summarised in Table 4.3. Overall, 21 adolescent controls were recruited [median age 17.0y (12.6, 18.4), 33% males, median BMI 19.1 (14.0, 35.7);Table 4.1], and 34 age and sex matched control data were selected from a previous study [median age 12.2y (7.7, 17.7), 59% males, median BMI 19.9 (13.5, 31.3);Table 4.1].

The IBD patient group was younger compared to adolescent controls (median 13.7 vs 17.0 years, $P < .0001$), had a higher percentage males (66% vs 33%, $P = .0320$), and (consequently) had lower systolic (median 107 vs 127 mmHg, $P < .0001$) and diastolic (median 62.5 vs 72 mmHg, $P < .0001$) blood pressure. BMI ($P = .1660$) and BMI z-score ($P = .4370$) showed no significant difference between groups. Comparison with an age-matched historic control group (n=34; previously studied by Shah et al., 2015) showed no significant difference in any demographic parameter compared to the present IBD cohort. All subjects included in the study (recruited or historic) were non-smokers.

	Healthy Controls (N=21)	IBD (N=35)	Historic Controls (N=34)	Control vs IBD *P* value	Historic Control vs IBD *P* value	Inactive disease (N=27)	Active disease (N=8)
Age at Study (yrs; range)	17.0 (12.6, 18.4)	13.7 (7.8, 16.5)	12.15 (7.7, 17.7)	***P* < .0001**	*P* = .371	13.8 (7.8, 16.4)	12.2 (10.0, 15.0)
Males (%)	33%	63%	59%	***P* = .032**	*P* = .808	63%	63%
Body Mass Index (kg/m^2)	21.8 (16.2, 27.0)	19.1 (14.0, 35.7)	19.9 (13.5, 31.3)	*P* = .166	*P* = .749	19.5 (14.0, 35.7)	17.9, (16.3, 22.8)
Body Mass Index z-score	0.43 (-2.41, 2.06)	0.11 (-2.23, 3.20)	0.42 (-1.93, 2.72)	*P* = .437	*P* = .846	0.11 (-2.23, 3.20)	0.14 (-1.61, 1.90)
Systolic BP	127 (104, 159)	107 (85, 135)	110 (90, 133)	***P* = .002**	*P* = .922	109 (85, 135)	104 (98, 125)
Diastolic BP	72 (61, 88)	62.5 (47,79)	61 (47, 80)	***P* < .0001**	*P* = .749	62.5 (47, 79)	59.5 (54, 75)
Age at Diagnosis of IBD (yrs)	--	10.9 (4.0, 15.9)	--	--	--	10.1 (4.0, 15.9)	11.4 (10.0, 14.2)
Time of study (yrs) after Diagnosis	--	1.18 (0, 10.7)	--	--	--	1.59 (0.09, 10.71)	0.73 (0, 1.31)

Table 4.1: Demographic data for IBD patients, healthy adolescent controls and historic age/sex matched controls from a previous study.

Historic controls were obtained from a previous study in Kawasaki disease (Shah et al., 2015). Results shown as median with range. Differences in gender proportion were assessed

by Fischer's exact test, other variables were assessed by Mann-Whitney U test.

Table 4.2 summarises routine clinical laboratory indices for patients and controls. IBD patients had lower HDL (1.20 vs 1.50, P = .0176) and higher LDL (2.29 vs 1.91, P = .0299) levels than controls. There was no significant difference in hs-CRP (P = .6046), SAA (P >.9999), non-fasting total cholesterol (P = .4627), VLDL (P = .6613) or triglycerides (P = .6951).

	Healthy Controls Median (range) N=21	IBD Patients Median (range) N=35	IBD Control vs Patients *P* Value
hs-CRP (mg/L)	0.54 (0.05, 7.91)	1.03 (0.04, 125.0)	*P* = .6046
SAA (mg/L)	2.88 (0.60, 21.7)	2.28 (0.49, 220.0)	*P* > .9999
Total Cholesterol (mmol/L)	3.7 (3.0, 5.4)	4.1 (2.6, 5.4)	*P* = .4627
LDL (mmol/L)	1.91 (1.42, 2.75)	2.29 (1.04, 3.35)	***P* = .0299**
HDL (mmol/L)	1.50 (0.80, 2.20)	1.20 (0.70, 2.50)	***P* = .0176**
VLDL (mmol/L)	0.39 (0.19, 0.94)	0.47 (0.20, 2.06)	*P* = .6613
Triglycerides (mmol/L)	0.85 (0.41, 2.06)	1.03 (0.45, 4.53)	*P* = .6951

Table 4.2: Acute phase proteins and lipid profile of study participants

High-sensitivity C-reactive protein (hs-CRP), serum amyloid A (SAA), total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), very low-density lipoprotein (VLDL) and triglycerides of study subjects. Differences between controls and patients assessed by Mann-Whitney U test.

Table 4.3 summarises treatment categories for patients by disease subtype. Patients could be on more than one treatment at the same time. The majority of all patients were on a purine analogue, predominantly azathioprine. A high proportion (75%) of UC patients were on mesalazine, while none in that subtype were on steroids. Biologic treatments consisted entirely of anti-TNF-α antibodies, namely the mouse-human chimeric monoclonal infliximab, and the fully humanised monoclonal adalimumab. Due to small numbers of participants in each treatment category, no statistical tests were performed to compare groups.

Treatment Type	Frequency in IBD (% of total 35)	Frequency in CD (% of total 20)	Frequency in UC (% of total 8)	Frequency in IBD-U (% of total 7)
Steroids	4 (11.4%)	3 (15.0%)	0 (0%)	1 (14.3%)
Biologics	7 (20.0%)	5 (25.0%)	1 (12.5%)	1 (14.3%)
Purine Analogues	27 (77.1%)	17 (85.0%)	3 (37.5%)	7 (100%)
Mesalazine	15 (42.9%)	4 (20.0%)	6 (75%)	5 (71.4%)
Feeds	4 (11.4%)	4 (20.0%)	0 (0%)	0 (0%)
Antibiotics	2 (5.7%)	1 (5.0%)	0 (0%)	1 (14.3%)
Methotrexate	3 (8.6%)	2 (10.0%)	1 (12.5%)	0 (0%)

Table 4.3: Treatments of paediatric IBD patients at time of recruitment.

Patients may be receiving more than one category of treatment. Biologics include anti-TNF antibodies infliximab and adalimumab. Purine analogues include azathioprine and mercaptopurine. Feeds include Modulen™ and Elemental.

5. Circulating Endothelial Cells in Paediatric IBD

5.1 Introduction

The healthy vascular endothelium lines all blood vessel walls attached to a basement membrane. In healthy individuals this is largely intact with very few cells sloughed off into the circulation. While previously thought of as a relatively passive selective barrier, this lining of cells has been understood more recently to be dynamic and active in response to numerous stimuli, and implicated in many aspects of vascular health and disease (Sabatier et al., 2009; Cahill and Redmond, 2016). While few endothelial cells circulate in health, when the vascular endothelium is injured either by disease or mechanically, cells detach from the basement membrane and increase the number of those in circulation. This has been demonstrated in vasculitis in the young (Clarke et al., 2010), acute myocardial infarction (Mutin et al., 1999) and pulmonary hypertension (Smadja et al., 2009). Additionally and more recently, it has been shown circulating endothelial cell (CEC) levels can be used as a prognostic aid in cancer (Yuan et al., 2015) (Ronzoni et al., 2010). This has been linked to CECs acting as marker for angiogenesis, with cells sloughing off from the basement membrane as tumours expand their vascularisation (Manzoni et al., 2015).

The mechanisms by which vascular endothelial cells detach are not fully elucidated, but appear to involve the mechanical shear forces of blood flow, atherosclerotic plaque formation, disruption of cell-cell interactions formed by integrins, and defective attachment to the extracellular basement membrane (Sabatier et al., 2009). It has been shown that CECs themselves are highly activated, and considered necrotic (Erdbruegger et al., 2006). CECs were initially noted over 40 years ago, when they were detected in peripheral blood smears on the basis of their presumed morphology (Gaynor et al., 1970). They were subsequently investigated in vascular diseases, but the fact they were defined by morphology under the microscope led to differing definitions susceptible to subjective assessment and requiring high

levels of expertise. This, combined with the need for permeabilisation of cells causing fragile cells to lyse, showed a need for a new, more reliable detection technique (Blann et al., 2005).

The low number of CECs in the blood (~1/mL blood for healthy adults) highlighted the need for a reliable marker for endothelial cells. This requirement led researchers to investigate the use of monoclonal antibodies to endothelial antigens. Novel monoclonal antibodies to new endothelial surface antigens (S-Endo 1 and HEC 19) were described and used to enumerate CECs (George et al., 1992; Sbarbati et al., 1991), while another group demonstrated an endothelial-specific antibody to S-endo 1, later characterised as CD146 (Bardin et al., 1996a; Bardin et al., 1996b). CD146 was shown to be expressed at a low level in smooth muscle cells and activated T-cells, whilst showing greater specificity for endothelial cells when compared to other markers, such as CD105 (found on activated leukocytes) and CD31 (found on a wide range of haematopoetic cells, such as platelets, monocytes and neutrophils) (Mutin et al., 1997; Blann et al., 2005). Since then, anti-CD146 antibodies combined with magnetic bead extraction to enrich CECs from whole blood, followed by counting under the microscope, has become the standardised consensus protocol for enumerating CECs, enabling a rigorous counting technique and for CEC values to be reliably compared across studies from different groups (Woywodt et al., 2006).

While it has been suggested epidemiologically that patients with IBD are at increased lifetime risk of coronary artery disease (Gandhi et al., 2012), myocardial infarction, stroke, and cardiovascular death (Kristensen et al., 2013; Fumery et al., 2014), there has been limited investigation into vascular endothelial injury. Garolla *et al* demonstrated a reduced number of circulating endothelial progenitor cells in IBD, which they hypothesised was due to increased levels of apoptosis caused by the disease. More generally, it has been shown in paediatric IBD that brachial artery flow-mediated dilation (FMD) is impaired, along with increased thickness of the carotid intima media (Aloi et al., 2012), indicating a general impairment of endothelial function. However no group has yet looked at levels of CECs in IBD, let alone a paediatric

population. A clear demonstration of endothelial injury, along with information on the levels of injury at varying severity of disease would provide invaluable insight into the cardiovascular risks posed to paediatric IBD patients, as well as the effectiveness of treatments in ameliorating those risks.

5.2 Methods

5.2.1 Patients

The participants used for this study are described in detail in Chapter 4. Briefly, 35 paediatric IBD patients were recruited [median age 13.7y (7.8-16.5), 63% males, median BMI 21.8 (16.2, 27.0)]: 20 CD, 8 UC and 7 IBD-U. All were classified by Physicians Global Assessment score as inactive (quiescent, n=27) or active (mild, n=8) IBD. 21 adolescent controls were recruited [median age 17.0y (12.6, 18.4), 33% males, median BMI 19.1 (14.0, 35.7)], and 34 age and sex matched control data were selected from a previous study [median age 12.2y (7.7, 17.7), 59% males, median BMI 19.9 (13.5, 31.3)] (Shah et al., 2015).

5.2.2 Circulating Endothelial Cell (CEC) Enumeration

CECs were enumerated using CD146-coated magnetic bead extraction from whole blood and then positively identified by *Ulex europaeus* Lectin-FITC conjugate staining as previously described in a consensus protocol (Woywodt et al., 2006). The protocol is detailed in Chapter 2.2.6.

5.3 Results

5.3.1 Comparing CECs levels of adolescent controls recruited in the present study versus historic paediatric controls

The 21 recruited adolescent controls were assessed for their CEC levels, but to increase the statistical power of the study, the possibility of using the CEC results from controls recruited from a previous study performed by our collaborators, Professor Brogan and colleagues (Shah et al., 2015) was explored. Sample collection and analysis for historic controls was performed by another researcher, though using the same protocol. 34 historic controls, age and sex-matched to the current IBD patients (Chapter 4), were compared to the 21 current adolescent controls (Figure 5.1). When assessing the difference in median CEC level by Mann-Whitney U test, there was no statistically significant difference between the two groups of controls (P = 0.485). This observation supported our decision to combine the recruited and historic controls (total N=55) to compare CEC levels with IBD patients.

5.3.2 Comparing CECs of paediatric IBD patients and healthy controls

CECs of pooled healthy controls were compared to all recruited IBD patients (Figure 5.2A). IBD patients showed significantly increased CECs (P <0.0001) compared to controls. CECs were still significantly increased when IBD patients were subdivided into those with inactive (P <0.0001) and those with active (P = 0.005) disease, though there was no significant difference between these categories of IBD (Figure 5.2B). When divided into disease subtype (Figure 5.2C), there was a similar outcome, with all categories being significantly increased compared to control (P = 0.0001 for CD, P = 0.026 for UC and P = 0.016 for IBD-U) but no IBD category being significantly different from each other.
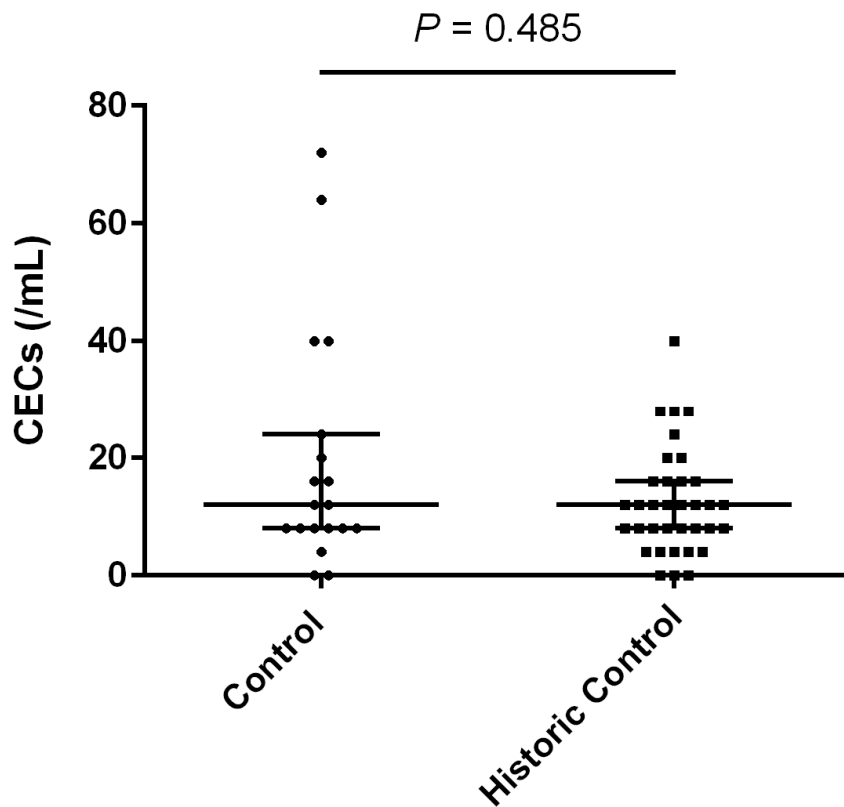
Figure 5.1: Comparing circulating endothelial cell (CEC) number in controls recruited in the current study versus historic controls.

CECs of healthy paediatric controls from the current study (N=21) were compared with healthy paediatric controls from a previous study, age and sex matched to the paediatric IBD patients recruited to the current study (N=34). Medians were compared with Mann-Whitney U test, with a non-significant difference (P = 0.485). Results shown as median with interquartile range.
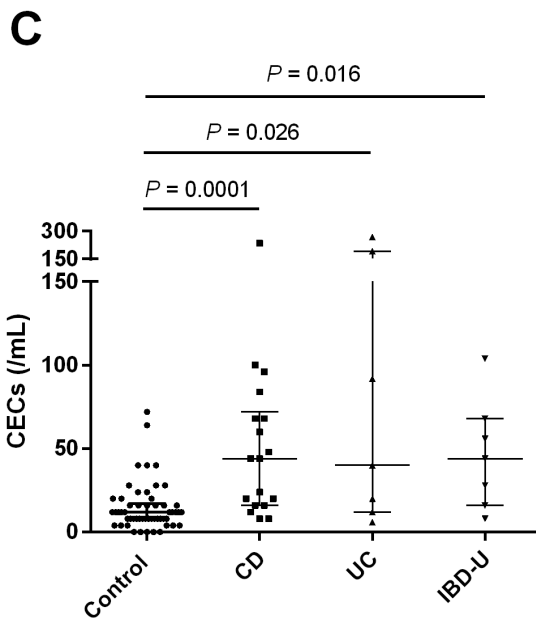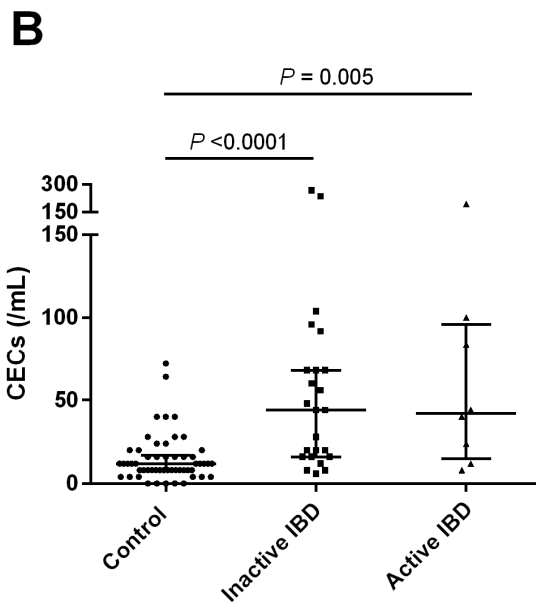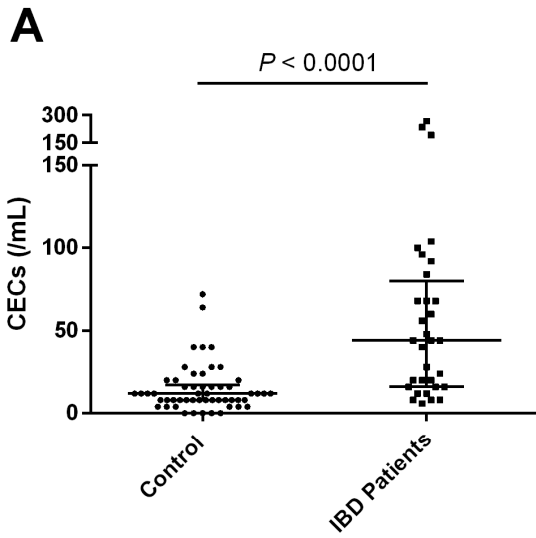
Figure 5.2: Comparing circulating endothelial cells (CECs) between paediatric inflammatory bowel disease (IBD) patients and healthy paediatric controls

(A) *CECs of pooled healthy paediatric controls from the current study (N=21) and a previous study (N=34) (Total N=55) were compared with paediatric IBD patients (N=35). Medians were compared with Mann-Whitney-U test, with a significant increase in IBD patients (P <0.0001).* *(B)* *CECs of the same pooled controls compared with the same paediatric IBD patients, subcategorised into inactive (N=27) and active (N=8) disease. Medians were compared with Kruskal-Wallis test with Dunn's multiple comparison.* *(C)* *CECs of the same pooled controls compared with the same paediatric IBD patients, subcategorised into the disease subtypes Crohn's disease (CD), ulcerative colitis (UC) and IBD-unclassified (IBD-U). Medians were compared with Kruskal-Wallis test with Dunn's multiple comparison. All results shown as median with interquartile range.*

5.3.3 Regression analysis of predictors for CECs

In order to analyse the possible confounding variables that could explain differences seen in CECs between controls and IBD patients, all variables must be normally distributed. When the frequency distribution of CECs was viewed (Figure 5.3A), a severe right skew was observed, with the mean value higher than the median value. However when CEC values were $\log_{10}$ transformed, their frequency distribution approximated a normal distribution (Figure 5.3B). Subsequently, all linear regression analyses were performed on $\log_{10}$ transformed CEC values. All other potential confounding variables were assessed for normality and all approximated the normal distribution (data not shown).

Lipid levels for historic controls were taken in a fasted state, whereas lipids for those recruited for the current study were taken as non-fasting. While non-fasting lipids have been shown to be just as effective for determining cardiovascular risk as fasting lipids (Sidhu and Naugler, 2012), it was decided this difference in sample acquisition meant only recruited controls should be used when looking at confounding variables (N=21) with linear regression modelling. Due to the large difference in median age between recruited adolescent controls and IBD patients (Chapter 4, P <0.0001), the correlation between age and $\log_{10}$ CECs was assessed (Figure 5.4). Recruits to the study showed a slight (R^2=0.10) negative correlation between age and $\log_{10}$ CECs, with younger participants having higher $\log_{10}$ CECs than older participants. The slope of the fitted regression line was significantly different from zero (P = 0.026), showing a significant relationship between age and $\log_{10}$ CECs.

To initially assess the effect of potential confounding variables on CEC number, individual unadjusted univariate linear regression analyses were performed for each potential confounder, with $\log_{10}$ CEC number as the dependent variable (Table 5.1). The only variables that significantly predicted $\log_{10}$ CECs were presence of IBD (P < 0.0001), age at investigation

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A

B

Figure 5.3: Frequency distributions of circulating endothelial cells (CECs) in paediatric inflammatory bowel disease (IBD) and healthy controls

(A) Histogram of CECs of pooled healthy paediatric controls from the current study (N=21) and paediatric IBD patients (N=35) (Total N=51, 5 excluded for not matching inclusion criteria) (B) Histogram of CECs of the same group of participants, after log$_{10}$ transformation.
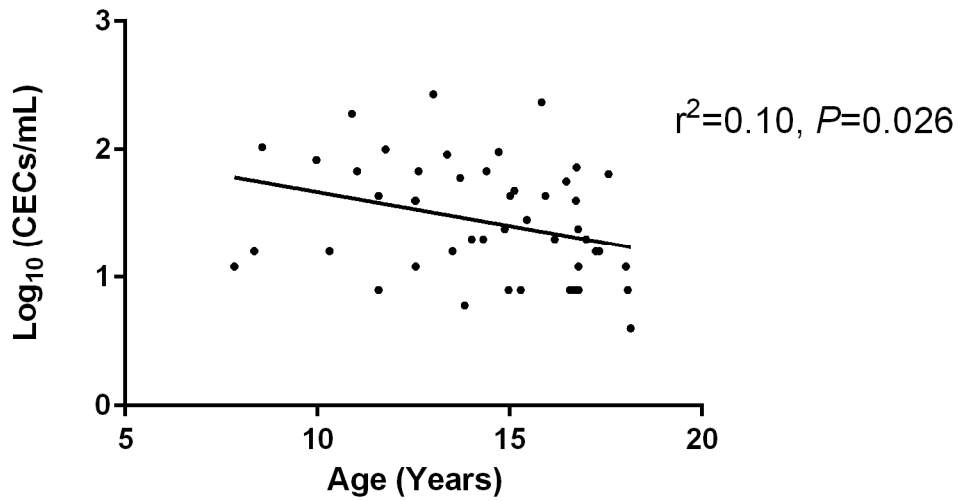
Figure 5.4: Correlation of log$_{10}$ circulating endothelial cells (CECs) and participant age in paediatric inflammatory bowel disease (IBD) and healthy controls

Scatter plot of log$_{10}$ CECs vs participant age for paediatric IBD patients and healthy adolescent controls (N=51). Correlation assessed by least-squares unweighted linear regression, with coefficient of determination R^2= 0.10, and the slope of the line significantly different from 0 (P = 0.026).

Variable	Unadjusted fold increase in median CEC (95% CI)	*P* value
Presence of IBD (IBD vs control)	3.23 (1.73, 6.05)	*P < 0.0001*
Age at Investigation (Years)	0.84 (0.75, 0.95)	*P = 0.006*
Presence of Inactive IBD	2.18 (1.13, 4.18)	*P = 0.021*
HDL (mmol/L)	0.44 (0.21, 0.95)	*P = 0.038*
Diastolic BP (mmHg)	0.96 (0.92, 1.00)	*P* = 0.061
Hs-CRP	1.00 (1.00, 1.00)	*P* = 0.120
Systolic BP (mmHg)	0.98 (0.96, 1.01)	*P* = 0.184
Presence of Active IBD	1.88 (0.72, 4.95)	*P* = 0.195
Serum Amyloid A	1.00 (1.00, 1.00)	*P* = 0.202
Males	1.53 (0.78, 3.03)	*P* = 0.211
VLDL (mmol/L)	1.90 (0.60, 6.07)	*P* = 0.270
Triglycerides (mmol/L)	1.34 (0.79, 2.29)	*P* = 0.271
BMI Z score	1.10 (0.86, 1.43)	*P* = 0.420
LDL (mmol/L)	1.26 (0.69, 2.32)	*P* = 0.443
Total Cholesterol (mmol/L)	0.95 (0.57, 1.60)	*P* = 0.848
Duration of Disease (Years)	1.01 (0.85, 1.21)	*P* = 0.873
Age of Diagnosis (Years)	1.00 (0.86, 1.17)	*P* = 0.969

Table 5.1: Univariate Predictors of circulating endothelial cell (CEC) counts.

Unadjusted univariate analysis for fold increase in median CEC count in association with presence of IBD, age at investigation, presence of inactive IBD, high density lipoprotein (HDL), diastolic blood pressure, high-sensitivity c-reactive protein (hs-CRP), systolic blood pressure, presence of active IBD, serum amyloid A, sex (male), very low density lipoprotein (VLDL), triglycerides, body mass index (BMI) z-score, low density lipoprotein (LDL), total cholesterol, duration of disease and age of diagnosis. Those with a significant P value are shown in bold.

(P = 0.006), presence of inactive IBD (P = 0.021) and HDL (P = 0.038). To fully assess which variables still significantly predicted $\log_{10}$ CEC number while taking into account all other variables, a multivariate linear regression model was created, taking into account all variables in Table 5.1. All variables were entered into a multivariate linear regression model, and then a backwards algorithm was performed. Through each iteration of the algorithm, the least significantly predictive variable was removed that had a predictive significance of P > 0.10. The overall change in R^2 of the model was also assessed to see if it had changed significantly from the previous iteration. The final model was found when either there were no more variables to remove which had predictive significance of P > 0.10, or removing a variable significantly changed the overall R^2 predictive power of the model. The final model once these conditions had been satisfied was:

$\log_{10}$ CECs = 1.06 + 0.509 * (presence of IBD), R^2 = 0.224, P < 0.0001

This showed that having IBD accounted for on average a $10^{0.509}$ = 3.23-fold increase in median CECs. It also showed that once presence of IBD had been accounted for, no other variable investigated significantly predicted CEC count.

5.4 Discussion

With IBD patients' increased lifetime risk of cardiac events (Gandhi et al., 2012; Kristensen et al., 2013; Fumery et al., 2014), it is imperative to find out the mechanisms and causes of this risk, so it can be addressed. The fact that endothelial dysfunction precedes many atherosclerotic changes to vessels (Davignon and Ganz, 2004) means markers for endothelial injury are ideal early warnings for prothrombotic risk in individuals. Being able to detect changes and injury to the vascular endothelium early on in disease progression would allow timely interventions to be started to maximise their effect.

In the paediatric cohort studied, we found significantly raised CECs in inactive as well as active IBD (Figure 5.2). The level of CECs did not seem to vary with disease activity or between disease subtypes, but was raised in all IBD patients. However, the confidence with which this can be said is reduced by small patient numbers, especially in the active disease category. The fact the only active disease patients recruited were those with mild disease is also a limiting factor. It may be that with more recruits, or with more severely active patients, a trend will be seen with patients with active disease having higher CECs than patients with inactive disease. The present study data however clearly suggests a raised level of CECs in patients even with inactive disease. This is important, as patients with inactive disease are currently considered in remission and well managed, whereas these CEC data suggest these patients may still have an ongoing subclinical pathological process in their vascular endothelium. An important future study would be to investigate changes in CEC number longitudinally over time, looking for correlations with disease activity. With increased patient numbers, patients could also be further stratified to determine if the kind of treatment they undergo for IBD affects their CEC levels, and therefore ongoing vascular injury. The wide range of treatments participants are on and small recruitment numbers make this analysis infeasible with the current study.

The wide range of healthy values that can be seen in CECs would normally reduce the sensitivity of this marker, but the heavy right skew to the distribution of values (Figure 5.3, the

vast majority of values are low, with a small minority high) means the chance of a high value being associated with IBD is actually increased compared to a normally distributed population. However, the skewed distribution of values means a transformation was required before linear regression could be applied, which assumes normally distributed values. A $\log_{10}$ transformation allowed uni and multivariate linear regression modelling to be carried out (Figure 5.3B). An initial hypothesis that IBD patients seemed to have higher CECs due to control participants being older than recruited IBD patients, rather than due to presence of IBD, was supported by the inverse correlation seen between age and $\log_{10}$ CEC number (Figure 5.4). However, when a multivariate linear regression model was made to predict $\log_{10}$ CEC number, the only variable with significant predictive power was the binary variable "presence of IBD". Once presence of IBD had been taken into account, no other variable, including age and conventional cardiovascular risk factors (Table 5.1) were significantly associated with CEC number.

One potential drawback to the consensus protocol for CEC enumeration (Woywodt et al., 2006) is the use of microscopy. Cells are counted manually, which is time consuming and also allows a degree of subjectivity. This was minimised but not eliminated by being blinded to disease activity when assessing CECs. Another possibility for counting CECs is flow cytometry, which is high-throughput and automated. This has predominantly been used to detect CECs in cancer (Malka et al., 2012; Yuan et al., 2015), and allows simultaneous detection of multiple surface markers, allowing subcategorization of cells. However, isolation methods, staining and categorisation of cells are not standardised, making comparisons between studies difficult. Flow cytometry also gives no information on cell morphology, increasing the chance of cells being misidentified. One method of circumventing these problems with flow cytometry has recently been described, by using an ImageStream™ flow cytometer (Samsel and McCoy, 2016). This allows simultaneous flow cytometric and imaging data to be acquired for each cell passing through the machine, allowing a high degree of specificity for CECs. While a relatively

new and expensive technique, in the future this could be used to automate CEC counting in a robust and reproducible way, allowing increased adoption in a clinical setting.

In summary, paediatric IBD patients show an increase in CEC number regardless of disease activity or subtype. These increases are present even when taking into account age and traditional cardiovascular risk factors. This suggests an ongoing subclinical endothelial injury is occurring associated with IBD, and could account for the increased cardiovascular risk IBD patients show over their lifetime. Further investigation in more severely active patients, and assessment longitudinally, would enable us to determine if these changes correlate with disease activity, and whether any IBD treatments are effective in reducing CEC numbers.

6. Microparticles in Paediatric IBD

6.1 Introduction

Microparticles (MPs) are vesicles of plasma membrane origin defined as being less than 1μm in diameter (Burnier et al., 2009), that circulate freely in the blood and are dynamically released and taken up by many cells (Ayers et al., 2015). Aspects of MP generation and the mechanisms involved have been explored in Chapter 3; this chapter will focus on MPs in disease.

MPs as a physiological phenomenon have come a long way from their original detection and characterisation as inert "platelet dust", to be considered an important, dynamic and systemic feature of health and disease, with a wide range of effects (Hargett and Bauer, 2013). While shown to be present in health, levels of circulating MPs are affected by stimuli that include mild hypoxia (Ayers et al., 2014), mild cardiac stress (Augustine et al., 2013) and cancer (Gong et al., 2015) to arthritis (Viñuela-Berni et al., 2015), diabetes (Jansen et al., 2016) and multiple sclerosis (Marcos-Ramiro et al., 2014). Due to their ability to encapsulate and deliver proteins (cytosolic and membrane bound) and micro (mi)RNA (Jansen et al., 2013) from cell to cell throughout the circulation, they can act to propagate inflammatory signals from a local source to throughout the body. This has been demonstrated in endothelial cells in Chapter 3 of this thesis. While the mechanism of MP release is not fully elucidated, their production is known to be distinct from exosomes, extracellular vesicles 40-150nm in diameter and derived from multivesicular bodies in the cell cytosol (Basu and Ludlow, 2016).

The most common detection method for characterising MPs is by flow cytometry, due to its high throughput of samples, and ability to characterise MPs by simultaneous multiple surface markers (Shantsila et al., 2014). Using flow cytometry, while convenient, does introduce certain biases. MPs are detected in flow cytometry by their light scattering, and the wavelength of light used to interrogate them (usually a 488nm laser) is larger than a proportion of the defined 150-1000nm range of MP sizes. This makes smaller MPs very hard to

detect and discern from background noise using flow cytometry, and increases the angle of scatter seen to the point where side scatter is a more accurate measure of MP resolution (Poncelet et al., 2016). Another problem established is the issue of event coincidence, where more than one MP passes the laser of the cytometer at once (Harrison and Gardiner, 2012). This can be minimised by reducing the flow rate of the cytometer to its lowest level. Improvements are however being made with regards standardising MP detection across different flow cytometry platforms, utilising bead-based gating (Cointe et al., 2016). While polystyrene beads show a higher refractive index than MPs, meaning detected MPs are larger in diameter than they appear (Edwin van der Pol et al., 2014), if the polystyrene beads used are kept the same across experiments, the size threshold will at least be consistent across experiments. Another method to enhance detection of small MPs is to use a fluorescence threshold rather than side scatter threshold when registering events (Arraud et al., 2015), but this requires a universal fluorescent MP marker. Due to exposed phosphatidylserine as part of MP production (see Chapter 3), it was originally thought that all MPs would be Annexin V$^+$. However, more recently an independent Annexin V$^-$ MP population has been identified (Connor et al., 2010). The reason for lack of Annexin V binding to these MPs is unclear, but highlights that when looking at Annexin V$^+$ events only, some of the MP population is potentially being excluded. A more sensitive phosphatidylserine binding protein is lactadherin, which has been shown to bind similar numbers of MPs to Annexin V, suggesting the Annexin V$^-$ populations seen really don't express phosphatidylserine (Latham et al., 2015).

Other methods of MP detection and enumeration are electron microscopy, dynamic light scattering and resistive pulse analysis (E. van der Pol et al., 2014). All of these methods allow higher resolution analysis of MP sizes, allowing more detailed size distributions to be determined, but none give the high throughput and flexibility of markers that can be used with flow cytometry. For this reason, flow cytometry is by far the most common form of MP analysis, and the most relevant in a clinical context.

The demonstrated increase in CECs in paediatric IBD shown in Chapter 5 indicate that IBD patients have an ongoing systemic vascular inflammation, even when considered as having inactive disease. The fact that MPs have been shown to be increased in many vascular diseases, including childhood stroke (Eleftheriou et al., 2012), systemic vasculitis (Eleftheriou et al., 2011) and in Kawasaki disease (Shah et al., 2015) would suggest IBD patients would also have increased circulating MPs. There have been limited studies of MPs in IBD, where they have been shown to be increased in adult CD patients (Leonetti et al., 2013). In an *in-vivo* murine model, injection of IBD MPs resulted in impairment in endothelium-dependent relaxation (Leonetti et al., 2013). Additionally, MPs isolated from paediatric IBD patients show an increase in thrombin generation (Deutschmann et al., 2013). Taken together, these studies indicate that an impact of IBD pathogenesis on cardiovascular health is likely. At present no detailed information is available on circulating MPs in paediatric IBD. In addition, evidence for vascular dysfunction in well-managed patients i.e. those in remission or with mildly active disease is unknown. Combined with the increased overall lifetime risk of cardiovascular events in IBD patients (Gandhi et al., 2012; Kristensen et al., 2013; Fumery et al., 2014), it is important to investigate not only the cellular origin of circulating MPs in paediatric IBD, but mediators of thrombosis such as tissue factor.

In summary, this study aimed to investigate differences in overall, platelet, endothelial, monocyte, neutrophil and tissue factor expressing MPs in paediatric IBD compared to controls using flow cytometry.

6.2 Methods

6.2.1 Study participants and sample collection

35 paediatric IBD patients and 21 healthy adolescent controls were recruited for the study, detailed in Chapter 4. All blood samples were obtained concurrently with other blood tests as part of participants' routine care, to prevent additional venepuncture. Blood samples were taken by trained paediatric phlebotomists, from the median cubital vein using a 21-gauge needle with butterfly extension directly into S-Monovette vacutainer tubes (Sarstedt). A tourniquet was used around the upper arm, and at least the first 5mL of blood was used for other routine hospital tests, to avoid contaminating study samples with endothelial cells dislodged from the puncture site. Each study participant had 4 x 1.4mL 3.2% sodium tricitrate tubes taken for MP analysis, which is detailed in Chapter 2.

6.2.2 MP gating, size resolution and staining on LSRII instrument

Antibody/fluorophore conjugates to cell markers used are detailed in Chapter 2.1.14, and the MP gating strategy along with optimisation of size resolution on the LSRII (BD, UK) flow cytometer are detailed in Chapter 2.1.15.

6.3 Results

6.3.1 Optimising Neutrophil MP staining

While CD11b is expressed on neutrophils, the fact that it is expressed at low levels on other myeloid cells such as monocytes and macrophages (Futosi et al., 2013) means a more specific neutrophil marker was sought. Carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM), otherwise known as CD66b, is known to be exclusively expressed on granulocytes and is used as a specific neutrophil marker (Scapini et al., 2016). However, during initial validation experiments on MPs derived from healthy adult plasma, all markers used could be compensated except for the neutrophil marker, CD66b. Neutrophils were isolated from healthy adult whole blood by dextran-ficoll gradient centrifugation, stimulated with phorbol 12-myristate 13-acetate (PMA) (Chapter 2) and stained with either CD11b-PerCP Cy5.5, or CD66b-PerCP Cy5.5. This was applied to healthy adult plasma MPs, with all samples additionally being stained with Annexin V-BV421 (Figure 6.1). While both neutrophil markers allowed identification of neutrophil MPs (NMPs) derived from stimulated neutrophils (Figure 6.1A, C, quadrant 2), CD66b showed practically no NMPs when used on a healthy adult plasma sample (Figure 6.1D, quadrant 2). Conversely, CD11b showed staining of NMPs in healthy adult plasma (Figure 6.1B, quadrant 2). Subsequent MP staining for neutrophils used CD11b-PerCP Cy5.5.
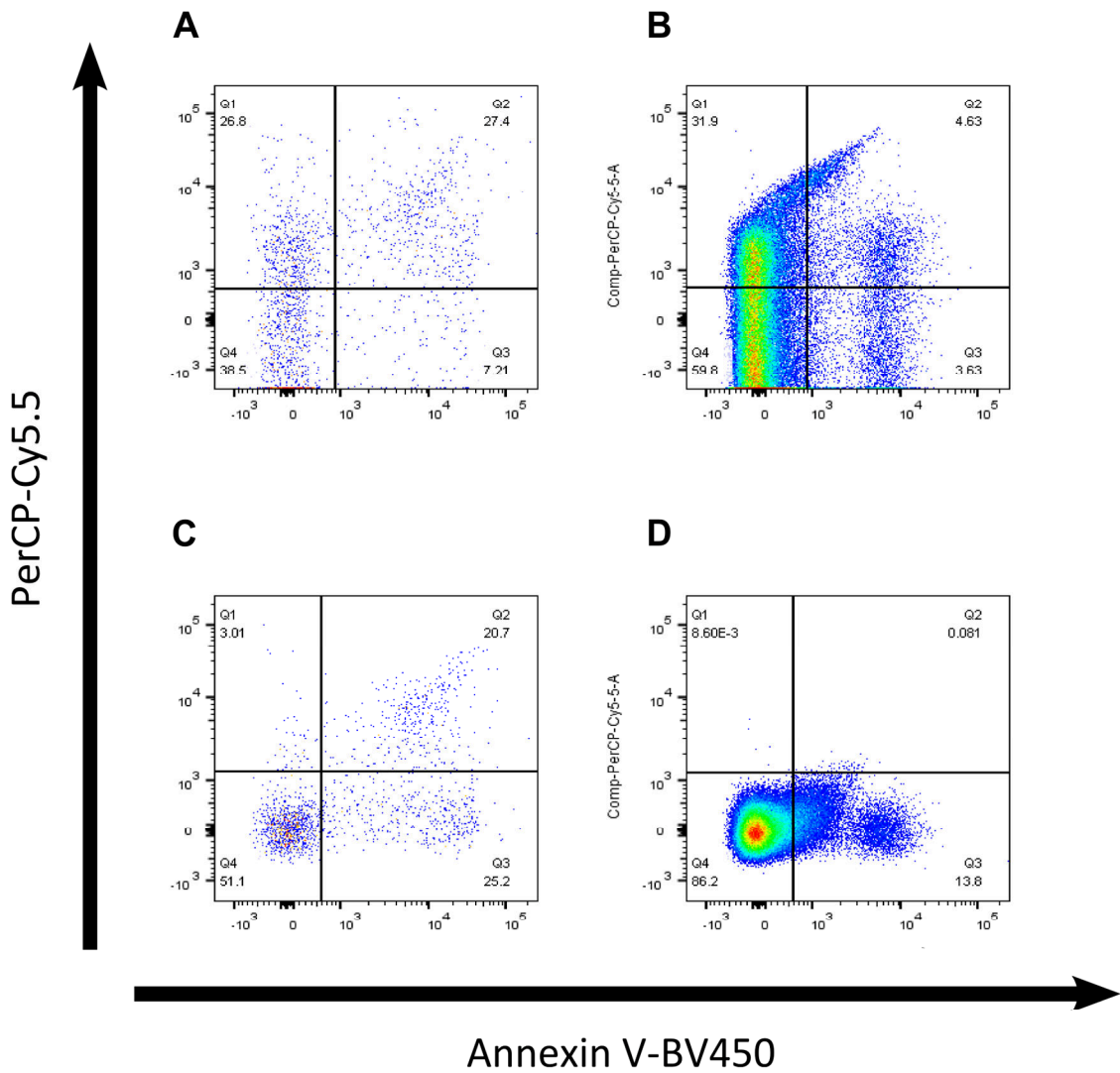
Figure 6.1: CD11b and CD66b as Neutrophil MP markers

2 x 10^6 neutrophils isolated from whole blood of a healthy adult donor were incubated in 1mL RPMI media and 2ng/mL phorbol 12-myristate 13-acetate (PMA) for 2h at 37°C to stimulate neutrophil MP (NMP) release. MPs were isolated from the supernatant, and also from healthy adult whole plasma, then stained with Annexin V-BV421, and either CD11b-PerCP Cy5.5, or CD66b-PerCP Cy5.5. MP preparations were run through an LSRII flow cytometer. (A) NMPs stained with CD11b-PerCP 5.5; (B) whole plasma stained with CD11b-PerCP Cy5.5; (C) NMPs stained with CD66b-PerCP Cy5.5; (D) whole plasma stained with CD66b-PerCP Cy5.5.

6.3.2   Setting gates for 7-marker MP flow cytometry Panel

To set positive and negative gates for Annexin V staining, healthy adult plasma MPs were resuspended in PBS pH7.4 rather than Annexin V buffer, to provide Ca^{2+}-free conditions (Figure 6.2A). All other gates were defined using aliquots of healthy adult plasma MPs resuspended in Annexin V buffer, singly stained with an isotype control of the same fluorophore. Figure 6.3 shows gates for CD42a (Figure 6.2B), CD62e (Figure 6.2C), CD142 (Figure 6.2D), CD14 (Figure 6.2E), CD144 (Figure 6.2F) and CD11b (Figure 6.2G).

Once these gates had been defined, they were used to define subcategories of MPs that denoted their cellular origins (Figure 6.3). All MPs were initially defined as <1μm in diameter by concurrent running of polystyrene beads, and additionally Annexin V^+ to differentiate them from small contaminants or electronic noise. CD42a$^+$ events were defined as platelet MPs, and the negative CD42a gate was also applied to all other subsets to help exclude false positives. Two endothelial markers were used (CD62e and CD144), along with a monocytic marker (CD14), a neutrophil marker (CD11b) and tissue factor (CD142). A subset of monocytic MPs was also defined, looking at the tissue factor expressing monocytic MPs (CD14$^+$, CD142$^+$).
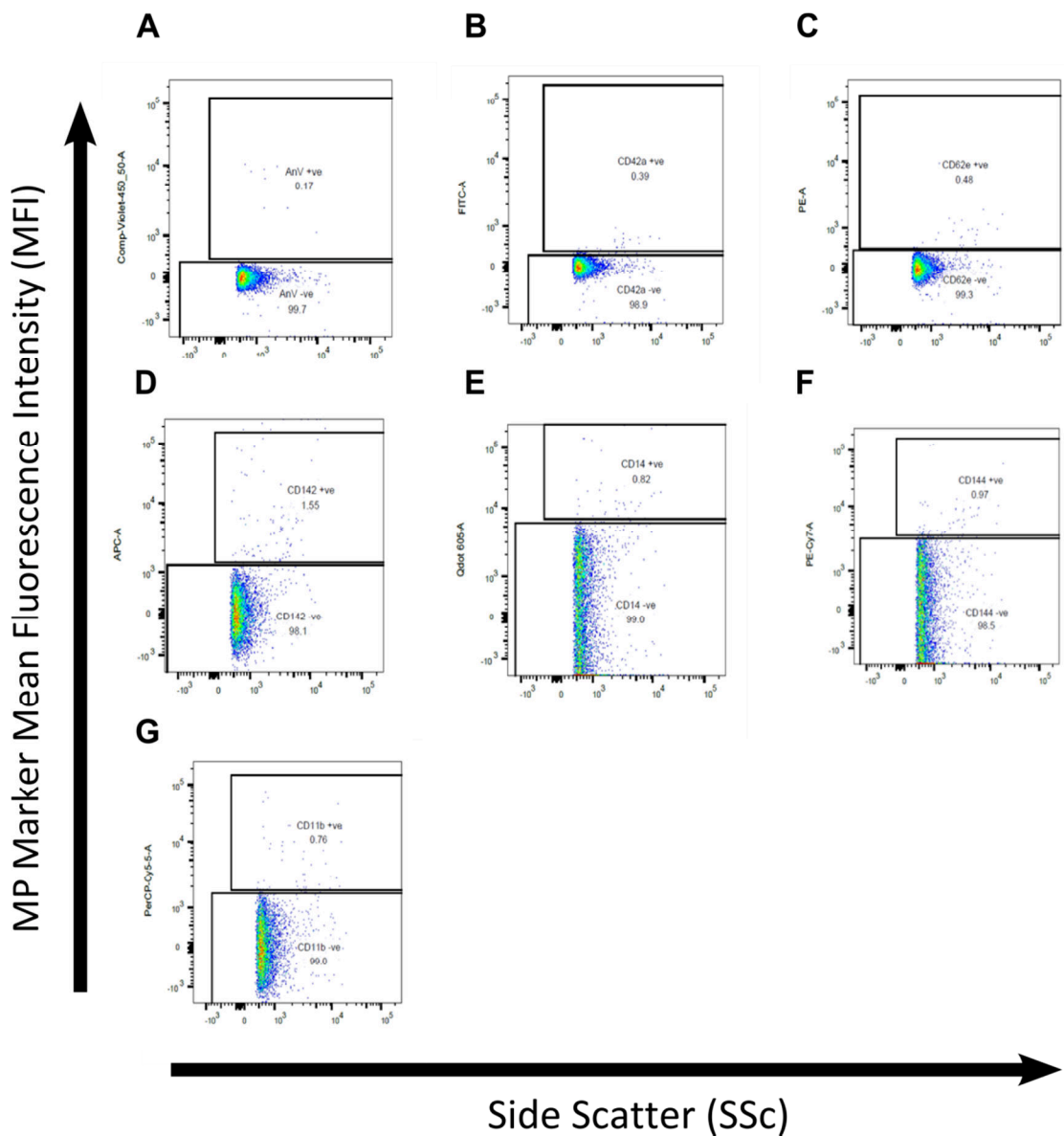
Figure 6.2: MP gates set for 7-marker panel using isotype controls/Ca^{2+}-free incubation

MPs were isolated from healthy adult whole plasma and used to define positive and negative gates for the 7 markers in the MP panel. IgG1 isotype controls were used for all markers, except for CD14 (IgG2a isotype control) and Annexin V (incubated in Ca^{2+}-free conditions, due to Ca^{2+} being needed for Annexin V binding to phosphatidylserine). Positive and negative gates for: **(A)** Annexin V-BV421; **(B)** CD42a-FITC; **(C)** CD62e-PE; **(D)** CD142-APC; **(E)** CD14-BV605; **(F)** CD144-PE Cy7; **(G)** CD11b-PerCP Cy5.5.
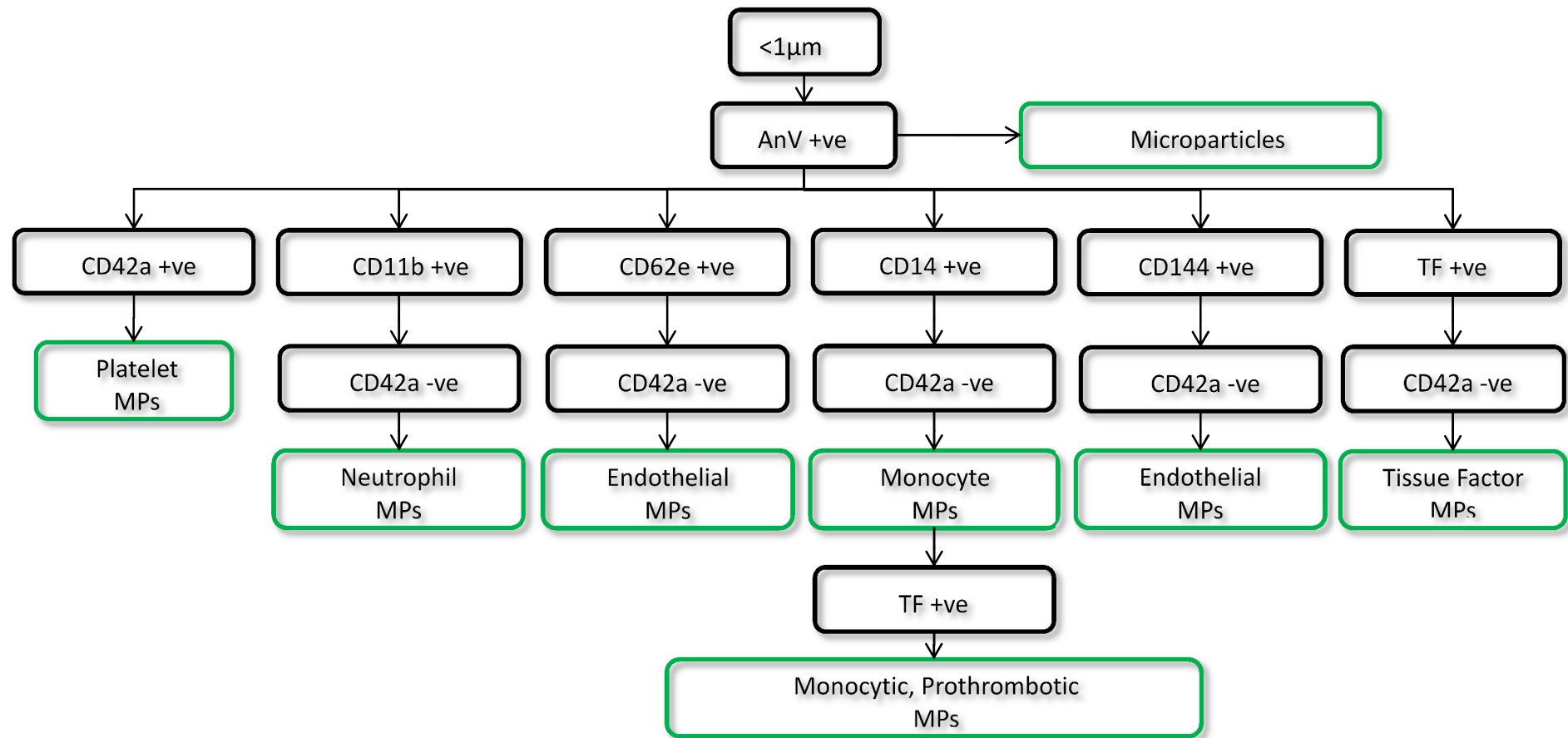
Figure 6.3: MP gating strategy flow diagram

A flow diagram detailing the subdivision of events detected by flow cytometry when analysing participant MP data. All MPs are defined as <1μm in diameter by side scatter compared to 1μm diameter polystyrene beads, and Annexin V^+. Subsequent subdivisions and cellular origins are determined by antibodies to surface markers. Due to platelet MPs being the most abundant in plasma, all non-platelet subdivisions include a CD42a negative gate to ensure their exclusion.

6.3.3 MP subsets in paediatric IBD patients and healthy adolescent controls

During initial data analysis of MP numbers, it was noted that no groups, subsets or individual participants showed significant CD144$^+$ events. These data were therefore excluded from analysis, and endothelial MPs were solely defined as CD62e$^+$. Overall Annexin V$^+$ MP numbers were no different when comparing healthy controls to IBD patients (Table 6.1, P = 0.232), and showed no differences within inactive or active disease subsets (P = 0.480). This was also reflected in platelet MPs with no difference between controls and IBD (P = 0.431) or within active and inactive disease (P = 0.704). CD11b$^+$ neutrophil MPs did show significantly increased numbers in IBD (P = 0.007, Figure 6.4B), though no significant difference between inactive disease and active disease (P >0.999). Similarly, CD62e$^+$ endothelial MPs showed significantly increased numbers in IBD patients (P =0.007, Figure 6.4A), and no significant difference between inactive disease and active disease (P >0.999). While CD14$^+$ monocytic MPs showed no significantly increased numbers in IBD (P = 0.052), there was a significant increase when comparing controls and active IBD (P = 0.032). Tissue factor, or CD142, MPs showed a significant increase in IBD (P = 0.001), with a trend to an increase between inactive and active IBD (Figure 6.4C). This trend was reflected in monocytic, tissue factor MPs, or CD14$^+$, CD142$^+$ MPs, with a significant increase in numbers in IBD (P = 0.001) and a trend to increase from inactive to active disease (Figure 6.4D).

Microparticles (/µL plasma)	Healthy Controls Median (range) N=21	All IBD Patients Median (range) N=35	IBD vs Controls P Value (95% CI of Diff)	Inactive IBD Median (range) N=27	Inactive vs Controls P Value (95% CI of Diff)	Active IBD Median (range) N=8	Active vs Controls P Value (95% CI of Diff)	P Value Kruskal Wallis
Total Annexin V	2908 (313, 19980)	4068 (543, 59614)	P = 0.232 (-1013, 3332)	3920 (543, 59614)	P = 0.285 (-1143, 3861)	5036 (994, 11478)	P = 0.374 (-2751, 4858)	P = 0.480
CD42a$^+$	292.5 (29, 2309)	364.4 (7, 5144)	P = 0.431 (-142, 350)	352.4 (7, 5144)	P = 0.542 (-166, 347)	574.6 (21, 2115)	P = 0.429 (-246, 749)	P = 0.704
CD11b$^+$, CD42a$^-$	128.9 (1, 2297)	604.4 (44, 8151)	**P = 0.007 (79, 654)**	590.4 (44, 7372)	**P = 0.022 (40.5, 589)**	977.2 (94, 8151)	**P = 0.018 (78.7, 1419)**	**P = 0.016**
CD62e$^+$, CD42a$^-$	135.6 (0, 5214)	1060 (21, 23965)	**P = 0.007 (121, 1473)**	843.1 (21, 23965)	**P = 0.012 (77.8, 1071)**	1815 (138, 8643)	P = 0.059 (-141, 1899)	**P = 0.024**
CD14$^+$, CD42a$^-$	523.2 (11, 4914)	1375 (97, 22576)	P = 0.052 (-19.9, 1515)	709.2 (97, 22576)	P = 0.146 (-165, 888)	1952 (226, 8587)	**P = 0.032 (150, 2027)**	P = 0.069
CD142$^+$	5.02 (0, 130)	45.3 (0, 5481)	**P = 0.001 (7.8, 206)**	27.9 (0, 5481)	**P = 0.008 (2.5, 92.5)**	338.9 (9, 1391)	**P < 0.0001 (109, 546)**	**P < 0.0001**
CD14$^+$, CD142$^+$, CD42a$^-$	3.05 (0, 127)	38.6 (0, 5004)	**P = 0.001 (3.6, 164)**	16.3 (0, 5004)	**P = 0.012 (1.6, 69.9)**	224.4 (4, 1252)	**P < 0.0001 (40.2, 298)**	**P = 0.001**

Table 6.1: Microparticles in paediatric IBD compared to healthy adolescent controls.

Microparticles (MPs) were extracted from peripheral blood by centrifugation and assessed by flow cytometry. MPs were defined as Annexin V positive and <1µm compared to polystyrene beads. Results shown as median with range. Differences between controls and all patients were assessed with Mann-Whitney U test, with Hodges-Lehman 95% confidence interval estimate. Differences between controls and quiescent and mild IBD were assessed by Kruskal-Wallis test with Dunn's multiple comparisons.
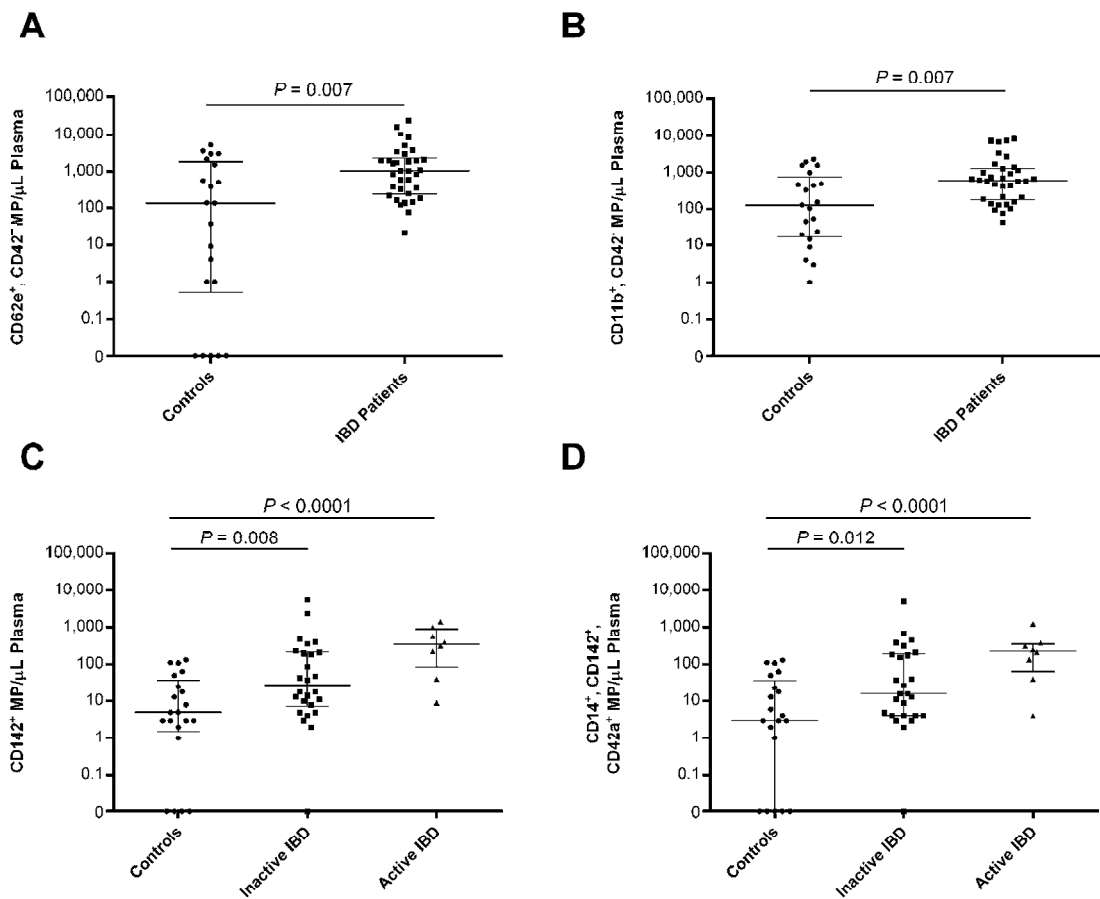
Figure 6.4: Characterisation of peripheral blood microparticles (MPs) in paediatric IBD compared to healthy adolescent controls.

MPs were isolated from paediatric IBD patients (n = 34) and healthy adolescent controls (n = 21). MPs were stained and defined as Annexin V$^+$ by flow cytometry, with subsets stained and defined as (A) CD62e$^+$, CD42a$^-$ endothelial MPs, (B) CD11b$^+$, CD42a$^-$ neutrophil MPs, (C) CD142$^+$ tissue factor MPs and (D) CD14$^+$, CD142$^+$, CD42a$^-$ monocytic, tissue factor MPs. Results presented with discontinuous logarithmic axes (in order to show zero values). Data shown as median with interquartile range, with differences assessed by (A, B) Mann-Whitney U test, or (C, D) Kruskal-Wallis test with Dunn's multiple comparisons. Endothelial and neutrophil MPs showed significant increases in paediatric IBD (both P = .007) compared to controls, with tissue factor MPs showing significant increases for inactive (n = 26) (P = .008) and active (n = 8) (P < .0001) paediatric IBD. Monocytic tissue factor MPs also showed significant increases for inactive (P = .012) and active (P < .0001) paediatric IBD.

6.3.4 Regression analysis of predictors for MP subset numbers

To gain an insight into the effect of confounding variables on MP number, univariate analysis of predictors of overall Annexin V[+] MPs was performed (Table 6.2). Of all potential predictive variables, the only that had significant linear predictive power was total cholesterol levels ($P = 0.025$).

Variable	Unadjusted fold increase in median Annexin V[+] MPs (95% CI)	*P* value
Total Cholesterol (mmol/L)	1.77 (1.08, 2.90)	***P = 0.025***
Diastolic BP (mmHg)	0.96 (0.92, 1.00)	*P* = 0.064
LDL (mmol/L)	1.76 (0.97, 3.21)	*P* = 0.064
Duration of Disease (Years)	0.86 (0.74, 1.01)	*P* = 0.073
Age at Investigation (Years)	0.90 (0.79, 1.01)	*P* = 0.075
VLDL (mmol/L)	2.48 (0.78, 7.91)	*P* = 0.121
Triglycerides (mmol/L)	1.49 (0.88, 2.51)	*P* = 0.136
Presence of IBD (IBD vs control)	1.64 (0.82, 3.26)	*P* = 0.157
Systolic BP (mmHg)	0.98 (0.96, 1.01)	*P* = 0.161
Presence of Inactive IBD	1.42 (0.72, 2.79)	*P* = 0.306
Presence of Active IBD	1.27 (0.48, 3.33)	*P* = 0.624
Age of Diagnosis (Years)	1.03 (0.89, 1.19)	*P* = 0.673
Serum Amyloid A	1.00 (1.00, 1.00)	*P* = 0.686
Hs-CRP	1.00 (1.00, 1.00)	*P* = 0.940
Males	0.99 (0.50, 1.96)	*P* = 0.977
HDL (mmol/L)	0.99 (0.45, 2.17)	*P* = 0.981
BMI Z score	1.00 (0.78, 1.29)	*P* = 0.990

Table 6.2: Univariate Predictors of Annexin V[+] MP counts.

Unadjusted univariate analysis for fold increase in median Annexin V[+] MP count in association with presence of IBD, age at investigation, presence of inactive IBD, high density lipoprotein (HDL), diastolic blood pressure, high-sensitivity c-reactive protein (hs-CRP), systolic blood pressure, presence of active IBD, serum amyloid A, sex (male), very low density lipoprotein (VLDL), triglycerides, body mass index (BMI) z-score, low density lipoprotein (LDL), total cholesterol, duration of disease and age of diagnosis. Those with a significant P value are shown in bold.

To fully assess which variables still significantly predicted each $\log_{10}$ MP subset while taking into account all other variables, a multivariate linear regression model was created for each MP subset, taking into account all variables in Table 6.3.

All variables were entered into a multivariate linear regression model, and then a backwards algorithm was performed. Through each iteration of the algorithm, the least significantly predictive variable was removed that had a predictive significance of $P > 0.10$. The overall change in R^2 of the model was also assessed to see if it had changed significantly from the previous iteration. The final models were found when either there were no more variables to remove which had predictive significance of $P > 0.10$, or removing a variable significantly changed the overall R^2 predictive power of the model. The final models once these conditions had been satisfied are shown in Table 6.4. CD42a$^+$, CD142$^+$ and CD142$^+$, CD14$^+$ subsets showed a suggestion of significant collinearity of variables in their final models, with one large positive variable counteracted by one negative variable. Due to this, the correlation between confounding variables was investigated with a Spearman's Rho matrix (Table 6.4).

Due to significant correlation between confounding variables, multivariate regression analysis was repeated for all subsets of MPs, with the following variables removed: HDL, LDL, VLDL, and diastolic blood pressure. Subsets that show a different model after collinear variables have been removed are shown in Table 6.5.

Log$_{10}$ (MP Subtype)	Overall model	R^2 of overall model	P value
Total Annexin V$^+$	2.143 + 0.375*(male) + 0.365*(cholesterol) + 0.283*(IBD)	R^2 = 0.282	P = 0.005
CD42a$^+$	**2.518 − 6.547*(triglycerides) + 14.340*(VLDL)**	**R^2 = 0.171**	**P = 0.026**
CD11b$^+$, CD42a$^-$	1.352 + 0.410*(cholesterol) + 0.940*(IBD)	R^2 = 0.375	P < 0.0001
CD62e$^+$, CD42a$^-$	3.203 − 0.021*(systolic BP) + 0.556*(cholesterol)	R^2 = 0.435	P < 0.0001
CD14$^+$, CD42a$^-$	2.333 + 0.430*(male) − 0.012*(systolic BP) + 0.527*(cholesterol) + 0.423*(IBD)	R^2 = 0.410	P = 0.001
CD142$^+$, CD42a$^-$	**2.959 − 6.920*(triglycerides) + 13.66*(VLDL) + 1.446*(IBD)**	**R^2 = 0.445**	**P <0.0001**
CD14$^+$, CD142$^+$, CD42a$^-$	**1.138 + 0.397*(cholesterol) − 7.806*(triglycerides) + 15.791*(VLDL) + 1.313*(IBD)**	**R^2 = 0.484**	**P <0.0001**

Table 6.3: Multivariate Predictors of MP subset counts.

Multivariate analysis for fold increase in median MP counts in association with presence of IBD, age at investigation, presence of inactive IBD, high density lipoprotein (HDL), diastolic blood pressure, high-sensitivity c-reactive protein (hs-CRP), systolic blood pressure, presence of active IBD, serum amyloid A, sex (male), very low density lipoprotein (VLDL), triglycerides, body mass index (BMI) z-score, low density lipoprotein (LDL), total cholesterol, duration of disease and age of diagnosis. All above variables were entered into an initial multivariate regression, then the backwards algorithm was iterated with exclusion P value >0.10. Overall model with significant predictors, R^2 of overall model, and significance of overall model shown. Models showing suggestion of collinearity shown in bold.

Spearman's Rho		Dia. BP (mmHg)	Chol. (mmol/L)	Trigly. (mmol/L)	VLDL (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
Sys. BP (mmHg)	Corr. Coeff.	**.586**	-.071	-.205	-.214	**.355**	-.235
	Sig. (2-tailed)	**.000**	.653	.187	.169	**.020**	.129
Dia. BP (mmHg)	Corr. Coeff.	1.000	-.155	-.301	-.300	.231	-.220
	Sig. (2-tailed)	.	.327	.053	.054	.141	.161
Chol. (mmol/L)	Corr. Coeff.		1.000	.191	.189	**.339**	**.769**
	Sig. (2-tailed)		.	.162	.166	**.011**	**.000**
Trigly. (mmol/L)	Corr. Coeff.			1.000	**.999**	**-.407**	.002
	Sig. (2-tailed)			.	**.000**	**.002**	.988
VLDL (mmol/L)	Corr. Coeff.				1.000	**-.410**	.004
	Sig. (2-tailed)				.	**.002**	.977
HDL (mmol/L)	Corr. Coeff.					1.000	-.019
	Sig. (2-tailed)					.	.889
LDL (mmol/L)	Corr. Coeff.						1.000
	Sig. (2-tailed)						.

Table 6.4: Spearman's Rho correlation matrix of significant confounding variables of MP number.

Correlation between systolic BP, diastolic BP, cholesterol, triglycerides, VLDL, HDL and LDL levels assessed by Spearman's Rho bivariate correlation matrix. Correlations with P < 0.05 shown in bold.

Log$_{10}$ (MP Subtype)	Overall model	R^2 of overall model	P value
CD42a$^+$	No model found	N/A	N/A
CD142$^+$, CD42a$^-$	4.802 − 0.130*(age) − 0.894*(triglycerides) + 0.911*(IBD)	R^2 = 0.465	P <0.0001
CD14$^+$, CD142$^+$, CD42a$^-$	4.739 − 0.147*(age) − 0.772*(triglycerides) + 0.735*(IBD)	R^2 = 0.440	P <0.0001

Table 6.5: Multivariate predictors of MP subset counts after removal of collinear confounding variables.

Multivariate analysis for fold increase in median MP counts in association with presence of IBD, age at investigation, presence of inactive IBD, high-sensitivity c-reactive protein (hs-CRP), systolic blood pressure, presence of active IBD, serum amyloid A, sex (male), triglycerides, body mass index (BMI) z-score, total cholesterol, duration of disease and age of diagnosis. High density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL) and diastolic blood pressure removed due to significant collinearity with other confounding variables. All above variables were entered into an initial multivariate regression, then the backwards algorithm was iterated with exclusion P value >0.10. Overall model with significant predictors, R^2 of overall model, and significance of overall model shown.

6.4 Discussion

Investigating differences in MP subsets between paediatric IBD and controls has shown that patients with IBD have increased circulating levels of CD62e$^+$ endothelial MPs, CD11b$^+$ neutrophil MPs, CD142$^+$ TF expressing MPs, and CD14$^+$, CD142$^+$ monocytic TF expressing MPs (Table 6.1,Figure 6.4). Both of the investigated subsets of TF expressing MPs also showed a trend to increase from inactive IBD to active IBD (Figure 6.4C,D), suggesting a correlation with disease activity. This corroborates the increased CECs seen in Chapter 5 and also provides an exciting, potentially more sensitive marker to disease activity, with differences seen between even inactive and mildly active disease in CD142$^+$ MPs. Not only is this marker more sensitive to disease activity, it also gives an insight into a possible cause for the proposed increased cardiovascular risk in IBD. With increased levels of CD142$^+$ MPs, IBD patients have a circulating increase in a known prothrombogenic factor (Mackman, 2004). This is in addition to the known catalytic feature of phosphatidylserine on the surface of MPs, that facilitates cleavage of prothrombin in the thrombin cascade (Lentz, 2003). This is covered in more detail in Chapter 7.

When taking into account confounding variables by regression analysis, presence of IBD appeared to increase all subsets of MPs except platelet MPs, from 1.92 x control in total Annexin V$^+$ MPs to 8.71 x control in CD11b$^+$ MPs (Table 6.4-5). Interestingly, the fact that all models included at least one other confounding variable (age, gender, cholesterol, triglycerides, systolic blood pressure) shows the overall balance of MPs circulating is sensitive to many different factors, and as many as possible should be accounted or controlled for to highlight changes in the variable of interest. This multifactorial sensitivity of MP levels makes it challenging to use MPs as a biomarker for a specific phenomenon in an individual patient, but means MP levels can be a very good biomarker for the overall effect of lifestyle, disease and genetic factors on a patient's vascular state. An important future experiment would be to follow patients longitudinally to see how MP levels change with progression of disease.

The patients recruited to this study created some drawbacks. The fact that only mildly active IBD patients were recruited means that no conclusions can be drawn about levels of MPs as the severity of IBD increases. This could be overcome either by recruiting inpatients who have moderate/severe disease, or to follow patients longitudinally and wait for remissive patients to relapse. This has the added benefit of providing baseline MP levels before the exacerbation. Small numbers of recruits mean that possible confounding factors of differing treatments could not be investigated. As well as insufficient recruits to investigate the effect of treatments, another possible source of bias in these results is the preferential detection of larger MPs, as detailed in 6.1. Steps were taken to minimise this bias, such as gating by side scatter for size, but running future samples through a more modern flow cytometer designed to detect smaller particles (Cointe et al., 2016) would help to minimise this bias further. Additionally, while numbers of MPs have been assessed, the overall functional effect of these MPs on coagulation state hasn't been addressed. This is explored in Chapter 7.

7. Thrombogenic Potential and Tissue Factor Pathway Activity in Paediatric IBD

7.1 Introduction

Emerging epidemiological evidence indicates that IBD patients may have an increased risk of thromboembolic events in their lifetime (Chapter 1). This, combined with observed increases in circulating endothelial cells (CECs) indicating vascular injury (Chapter 5), and increases in circulating MPs, particularly those expressing TF (Chapter 6), collectively suggest that paediatric IBD patients may be in a chronic prothrombotic state due to these ongoing inflammatory insults.

One method to assess the overall thrombotic state in a subject is to assess thrombin generation. Since the production of thrombin is reliant on the interplay of several different upstream proteins and their respective activities (Al Dieri et al., 2012), looking at the final outcome of all of these interactions, namely the production of thrombin, allows an overview into the coagulation state of the subject (Campo et al., 2012). The ability to measure thrombin production in real time reproducibly however has only recently been achieved, and relies on a calibrated automated fluorogenic thrombogram (Hemker et al., 2002; Hemker et al., 2004). Previous methods of detecting thrombin production required aliquots to be taken of the sample at multiple time points which was laborious, required large sample volume and was prone to error. The fluorogenic substrate used in the calibrated automated thrombogram (CAT) allows near continuous monitoring of thrombin production in a single sample without taking aliquots. When run concurrently with a calibrator of known thrombin concentration, the rate of change in fluorescence can be converted into an absolute value of thrombin produced between time points. This methodology has been used in the current study to assess differences in thrombin production between different patient groups with far greater ease than ever before (Eleftheriou et al., 2011).

Thrombosis is thought to be initiated *in vivo* when TF, from the disrupted endothelium or monocytes, activates factor VII (FVII) forming a complex (van Veen et al., 2009). This complex causes an initial production of thrombin via the prothombinase complex, but is insufficient to produce fibrin and a clot. However, this thrombin then combines with and activates the upstream effectors factor V (FV), factor VIII (FVIII) and factor XI (FXI), causing positive feedback through the thrombin cascade and production of thrombin and fibrin (Figure 7.1; Gailani and Broze, 1991). The action of thrombin on these upstream factors, and the combination of FV and factor Xa (FXa) to produce the prothombinase complex are in fact catalysed and potentiated by exposed PS (Lentz, 2003). While this is exposed on the surface of activated platelets and is a major driver of coagulation, as evidenced by those with Scott syndrome who cannot expose PS (Castoldi et al., 2011), PS is also exposed on the surface of MPs, providing a potential mechanism for MPs to be prothrombotic in themselves regardless of cellular origin. Indeed, multiple studies have shown MPs to be prothrombotic, whether isolated from endothelial cells (Combes et al., 1999), (Davizon and López, 2009) or in young patients with high cardiovascular risk and subclinical atherosclerosis (Suades et al., 2015). TF on its own is in an inactive, or encrypted state. However, TF in close proximity with PS promotes its conversion into an unencrypted or active state, fully realising the potential of TF to promote coagulation (Spronk et al., 2014). This further shows how MPs can act as an ideal candidate for promoting thrombosis due to the presence of exposed PS and TF on their surface. While studies in IBD have been limited in this area, Deutschmann *et al* demonstrated increased endogenous thrombin potential (ETP) in plasma in paediatric IBD, which corresponded with increased circulating MPs as assessed by ELISA (Deutschmann et al., 2013).

While it is important to assess the overall thrombin generation potential of paediatric IBD patients to help identify potential thrombogenic risk, the fact that increased TF-expressing MPs have also been found (Chapter 6) warrants further investigation into TF activity. However,
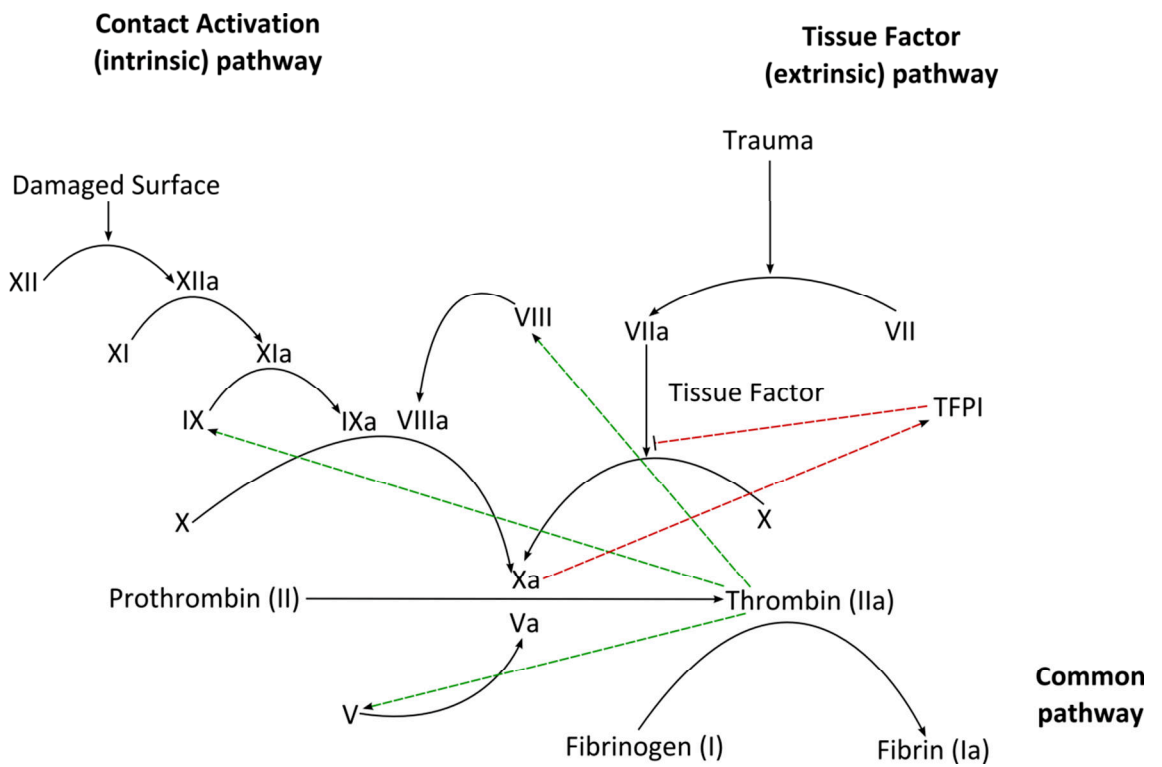
Figure 7.1: The thrombin cascade and effect of Tissue Factor Pathway Inhibitor (TFPI) on the extrinsic pathway

An overall representation of the thrombin generation cascade. Positive feedback is denoted by green arrows, negative feedback by red arrows. Adapted from a diagram by (Pallister et al., 2011)

it is important to note that TF does not operate alone. Tissue factor pathway inhibitor (TFPI) is active as a homodimer and inhibits the action of TF primarily by binding to FXa and then competitively inactivating the TF:FVIIa complex via a quaternary complex (Figure 7.1; Peraramelli et al., 2014). The fact that TFPI works in tandem with other coagulation cascade factors to competitively inhibit the action of TF means that the action of TF is highly sensitive to the levels of active TFPI expressed at any one time. Under normal conditions, TFPI is expressed on the endothelium of the microvasculature, ostensibly to prevent coagulation in the small vessels (Lupu et al., 1997). Under pathologic conditions, TFPI has also been shown to be expressed on adherent monocytes and macrophages (Osterud et al., 1995). The fact that TFPI expression changes in pathological conditions and its central role in the inhibition of the action of TF means that investigating the activity of only TF would not provide a complete picture, and both must be simultaneously investigated to provide information on the coagulation state of the subject.

In this study, we aimed to investigate the PPP and MP-mediated thrombin generation parameters of paediatric IBD patients, and simultaneously their TF and TFPI activities, to help gain an understanding into their thrombotic potential and hopefully explain their increased lifetime risk of thrombotic events.

7.2 Methods

7.2.1 Participants and Sample Collection

The participants used for this study are described in detail in Chapter 4. Briefly, 35 paediatric

IBD patients were recruited [median age 13.7y (7.8-16.5), 63% males, median BMI 21.8 (16.2,

27.0)]: 20 CD, 8 UC and 7 IBD-U. All were classified by Physicians Global Assessment score as

inactive (quiescent, n=27) or active (mild, n=8) IBD. 21 adolescent controls were also recruited

[median age 17.0y (12.6, 18.4), 33% males, median BMI 19.1 (14.0, 35.7)].

7.2.2 Thrombin Generation Assay (TGA)

Citrated plasma and isolated MPs were isolated from study subjects, and the thrombin

generation assay was performed on samples as per Chapter 2.1.17. 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.

7.2.3 Tissue Factor (TF) Activity Assay

TF activity in citrated PPP was assessed by a commercial chromogenic kit (Abcam, UK).

Samples were thawed in a 37°C water bath and then diluted 1 in 2 with the provided sample

diluent. Standards were prepared as per kit instructions, creating a dilution series of 0-250

pmol/L. A stoichiometric excess of factor VIIa (FVIIa) and factor X (FX) were mixed by pipette

(50µL assay diluent + 10µL FVIIa + 10µL FX per reaction) and then 70µL added per well to a

clear, flat bottomed 96-well plate. 10µL of sample or standard was added per well and

incubated at 37°C for 30min to allow the action of sample TF/FVIIa complex to cleave FX to

FXa. After incubation, FXa activity was monitored by adding 20µL of a substrate specific to FXa,

which when cleaved produces a chromophore that absorbs at 405nm. 20µL of FXa substrate

was added and the mix incubated at 37°C, with absorbance at 405nm measured immediately

and at 5min intervals for a total of 30min, monitored by a Multiskan EX plate reader (Thermo

Electro Corp, UK). Samples were compared to a known calibrator supplied in the kit to

determine TF activity equivalent to a known concentration of TF.

7.2.4 Tissue Factor Pathway Inhibitor (TFPI) Activity Assay

TFPI activity in citrated PPP was assessed by a commercial chromogenic kit (Sekisui

Diagnostics, Japan) based on a similar principle as the TF assay described. TF, FVIIa, and FX

were added, with a FXa specific chromogenic substrate used to follow the activity of TF/FVIIa

complex to cleave FX. Sample TFPI activity was derived from its ability to inhibit the action of

the TF/FVIIa complex on FX. Comparison with a standard of known TFPI activity, provided in

the kit, allowed an absolute measurement of TFPI activity. Samples and calibrators were

diluted 1 in 20 with the provided TFPI-depleted plasma, and 20µL of each were incubated with

20µL of provided TF/FVIIa complex for 30min at 37°C. 20µL of FX was added and incubated for

15min at 37°C, before the addition of 20µL EDTA solution and 20µL FXa substrate. After 5min,

50µL glacial acetic acid was added followed by absorbance recording at 405nm. TFPI

concentrations were interpolated directly from the standard curve, and multiplied by 20 to obtain an undiluted value.

7.2.5 Effect of transporting samples on ice on thrombin generation

To assess the quantitative effect of cold activation of the coagulation cascade (Engbers et al., 2012) on measured thrombin production, blood was taken from 3 healthy adult donors via the antecubital vein into sodium citrate tubes (Sarstedt, UK) and submitted to the following conditions, with 1mL of whole blood for each condition. Control samples were kept at room temperature or on ice for 1h and then processed into platelet poor plasma (PPP) as per Chapter 2. Stimulated samples were both incubated at room temperature for 1h after addition of the calcium ionophore A23187 (Sigma, UK) to a final concentration of 10nM to promote microparticle formation. One sample was then kept on ice, one sample at room temperature, for 1h and then processed into PPP as per Chapter 2. Unstimulated samples were processed the same as stimulated samples, without the addition of A23187. Frozen PPP samples were thawed in a 37°C water bath and then split into 2 x 200μL aliquots. One aliquot was assessed by TGA as described above, while the other was processed to isolate MPs and then assessed by TGA.

7.3 Results

7.3.1 Effect of transporting samples on ice versus room temperature on thrombin generation

When running the provided TGA calibrator (Pathway Diagnostics, UK), a linear dilution series was demonstrated at thrombin concentrations up to 450nM (Figure 7.2, R^2 = 0.99, P < 0.0001), indicating the assay had been set up correctly.

Due to logistical considerations, experimental samples were transported on ice. To determine if this had an effect on thrombin generation, healthy adult volunteer blood was assessed as per 7.2.5. When assessing the effect of sample transport on ice on kinetic parameters of thrombin generation, whether looking at whole plasma or MP-mediated thrombin generation, there were no significant differences in any parameter under any conditions between incubation at room temperature or on ice (Figure 7.3-4). In addition, when looking at individual donors there was no consistent trend to increase or decrease in any parameter, i.e. not in any instance or any parameter did all three donors increase, or all three donors decrease their values when incubated on ice. There were however trends between conditions, with stimulated compared with control or unstimulated showing a decrease in lag time (Figure 7.3A, 4A), increase in peak height (Figure 7.3B, 4B), decrease in peak time (Figure 7.3C, 4C), increase in velocity index (Figure 7.3D, 4D) and increase in endogenous thrombin potential (Figure 7.3E, 4E).
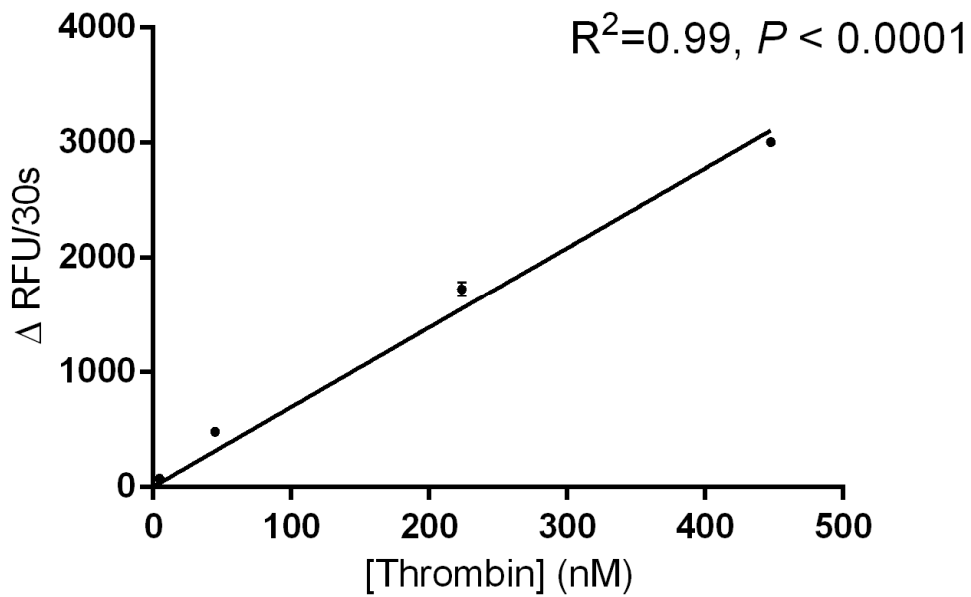
Figure 7.2: Thrombin Generation Assay (TGA) Calibration Curve

A Technothrombin™ calibrator of known concentration of thrombin was diluted serially 1 in 2 to create a dilution series and run through the thrombin generation assay (TGA) protocol in duplicate. Average change in relative fluorescence intensity (RFU) per 30s was assessed and plotted against the known concentration of thrombin in the sample. Responses demonstrated a positive linear relationship with slope significantly different from zero, assessed by least-squares linear regression (R^2 = 0.99, P < 0.0001). This linear regression line was used to interpolate production of thrombin for all samples based on change in RFU.

A

Lag Time

B

Peak Height

C

Peak Time

D

Velocity Index

E

AUC
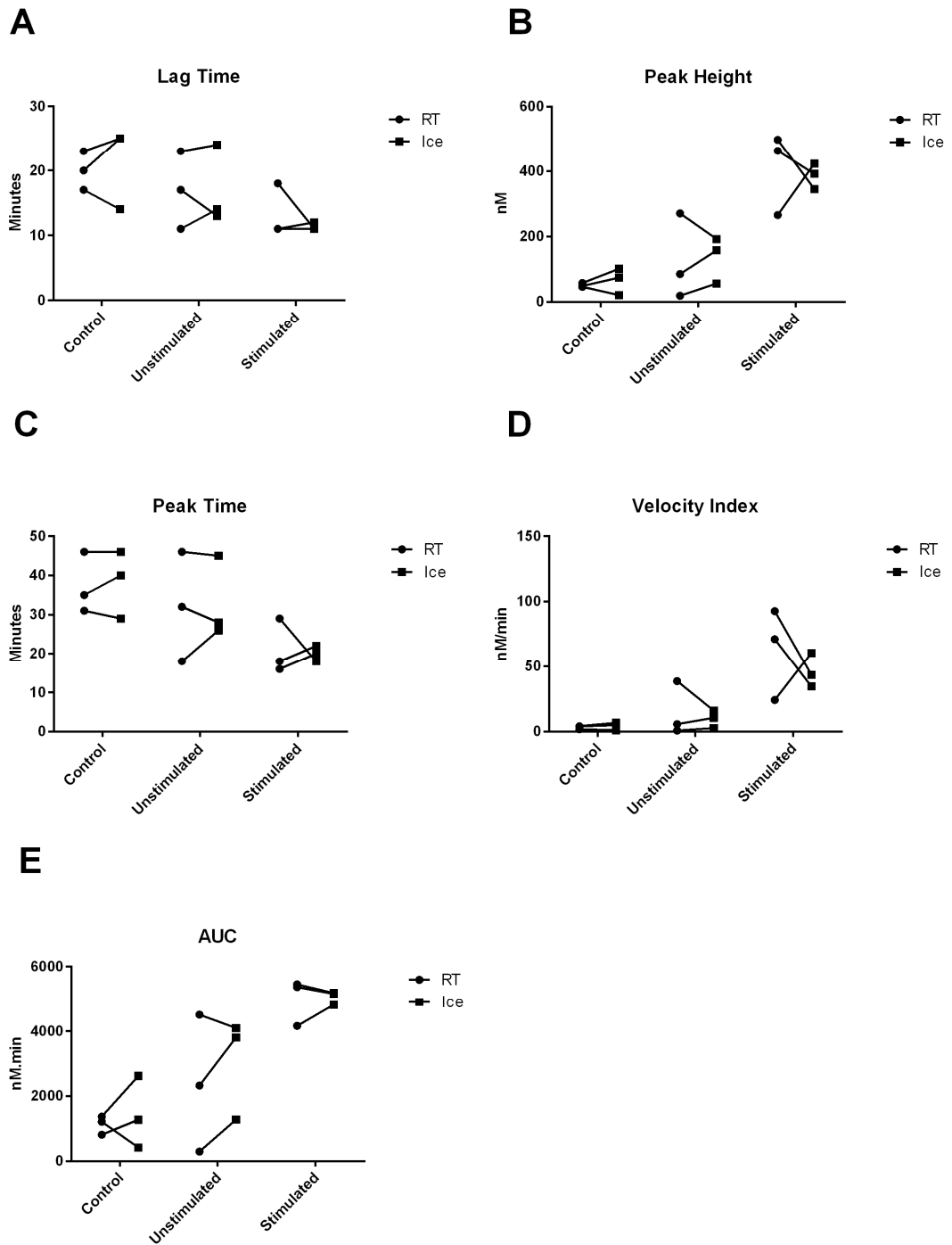
Figure 7.3: Effect of ice transport on whole plasma thrombin generation

Citrated whole blood from healthy adult donors (N=3) was subjected to the following conditions: control

samples were left at room temperature or on ice for 1h. Stimulated samples were incubated with 10nM

A23187 at room temperature for 1h, and then incubated for a further 1h at room temperature or on ice.

Unstimulated samples were treated the same as stimulated samples with the absence of A23187. All

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samples were then centrifuged twice at 5000 x g for 5min to obtain platelet poor plasma, and run

through the thrombin generation assay (TGA) protocol in duplicate. The following parameters were

assessed: (A) time taken for initial thrombin to be produced (min), (B) Peak thrombin produced (nM), (C)

time taken for peak thrombin to be produced (min), (D) rate of initial thrombin production (nM/min) and

(E) endogenous thrombin potential or area under the curve of the thrombogram (nM.min). All

differences between room temperature and ice incubation were assessed with 2-way ANOVA with

Sidak's post-hoc multiple comparisons. No means of any conditions in any parameter were significantly

different (P > 0.05).
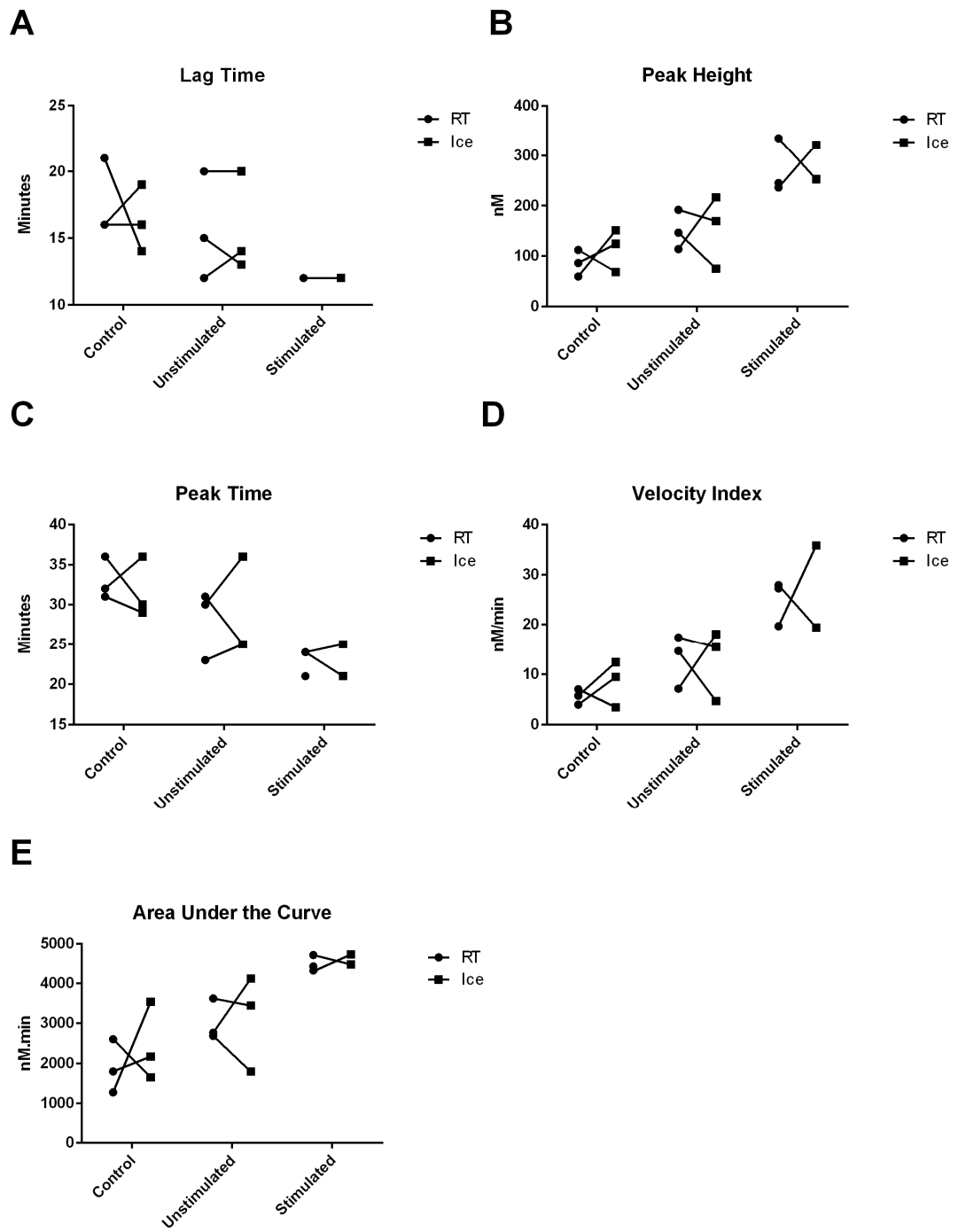
Figure 7.4: Effect of ice transport on MP-mediated thrombin generation

Citrated whole blood from healthy adult donors (N=3) was subjected to the following conditions: control

samples were left at room temperature or on ice for 1h. Stimulated samples were incubated with 10nM

A23187 at room temperature for 1h, and then incubated for a further 1h at room temperature or on ice.

Unstimulated samples were treated the same as stimulated samples with the absence of A23187. All

samples were then centrifuged twice at 5000 x g for 5min to obtain platelet poor plasma, and then

*centrifuged at 20,000 x g for 1h to isolate microparticles (MPs). The supernatant was aspirated leaving 20μL and the MP pellet, which was resuspended in MP-free plasma and run through the thrombin generation assay (TGA) protocol in duplicate. The following parameters were assessed: **(A)** time taken for initial thrombin to be produced (min), **(B)** Peak thrombin produced (nM), **(C)** time taken for peak thrombin to be produced (min), **(D)** rate of initial thrombin production (nM/min) and **(E)** endogenous thrombin potential or area under the curve of the thrombogram (nM.min). All differences between room temperature and ice incubation were assessed with 2-way ANOVA with Sidak's post-hoc multiple comparisons. No means of any conditions in any parameter were significantly different (P > 0.05).*

7.3.2 Comparing TGA parameters between paediatric IBD patients and healthy controls

Paediatric IBD patients showed no significant difference in peak thrombin, velocity index, or endogenous thrombin potential for either MP-mediated or whole plasma thrombin generation compared to controls (Table 7.1). However, paediatric IBD patients did show increased lag time and time to peak thrombin generation compared to controls, whether MP-mediated (Figure 7.5A,B) or in whole plasma (Figure 7.5C,D).

	Healthy Controls Median (range) N=21	IBD Patients Median (range) N=35	IBD vs Controls P value (95% CI of Diff)
MP Lag Time (min)	20 (10.5, 34)	28.5 (17, 43.5)	*P = .001 (3, 11)*
MP Peak Thrombin (nM)	118.2 (19.4, 488.8)	87.4 (11.3, 346)	*P = .182 (-86.2, 12.6)*
MP Peak Time (min)	32 (20, 44.5)	43 (25, 64)	*P < .0001 (5.0, 15.5)*
MP Velocity Index (nM/min)	5.9 (0.5, 46.8)	5.4 (0.4, 49.4)	*P = .749 (-2.78, 2.90)*
MP Endogenous Thrombin Potential (nM.min)	2602 (216, 4895)	1976.5 (136, 5356)	*P = .161 (-1310, 223)*
Plasma Lag Time (min)	21 (12, 39)	31 (15, 74)	*P < .0001 (6.0, 15.5)*
Plasma Peak Thrombin (nM)	136.3 (43.4, 427.2)	116.3 (2.3, 345.9)	*P = .093 (-74.7, 9.1)*
Plasma Peak Time (min)	32 (20.5, 55.5)	44 (19, 88)	*P = .002 (3.5, 16.5)*
Plasma Velocity Index (nM/min)	7.9 (0.8, 37.3)	11.1 (0.2, 40.2)	*P = .264 (-1.44, 7.60)*
Plasma Endogenous Thrombin potential (nM.min)	3111 (1298, 4920)	2687 (2, 5776)	*P = .209 (-1107, 273)*

Table 7.1: Kinetics of Thrombin generation in isolated microparticles (MPs) and plasma of paediatric IBD compared to healthy adolescent controls.

MPs isolated by centrifugation were resuspended in pooled healthy MP-depleted plasma, and assessed along with platelet poor plasma (PPP) for thrombin generation kinetics by a fluorogenic thrombin generation assay (Pathway Diagnostics). Parameters assessed were: lag time for initial thrombin generation, time to peak thrombin generation, velocity index, or initial rate of thrombin generation, and area under the curve, or total thrombin produced. Results shown as median with range. Differences between controls and patients were assessed with Mann-Whitney U test, with Hodges-Lehman 95% confidence interval estimate.

A

B

C

D
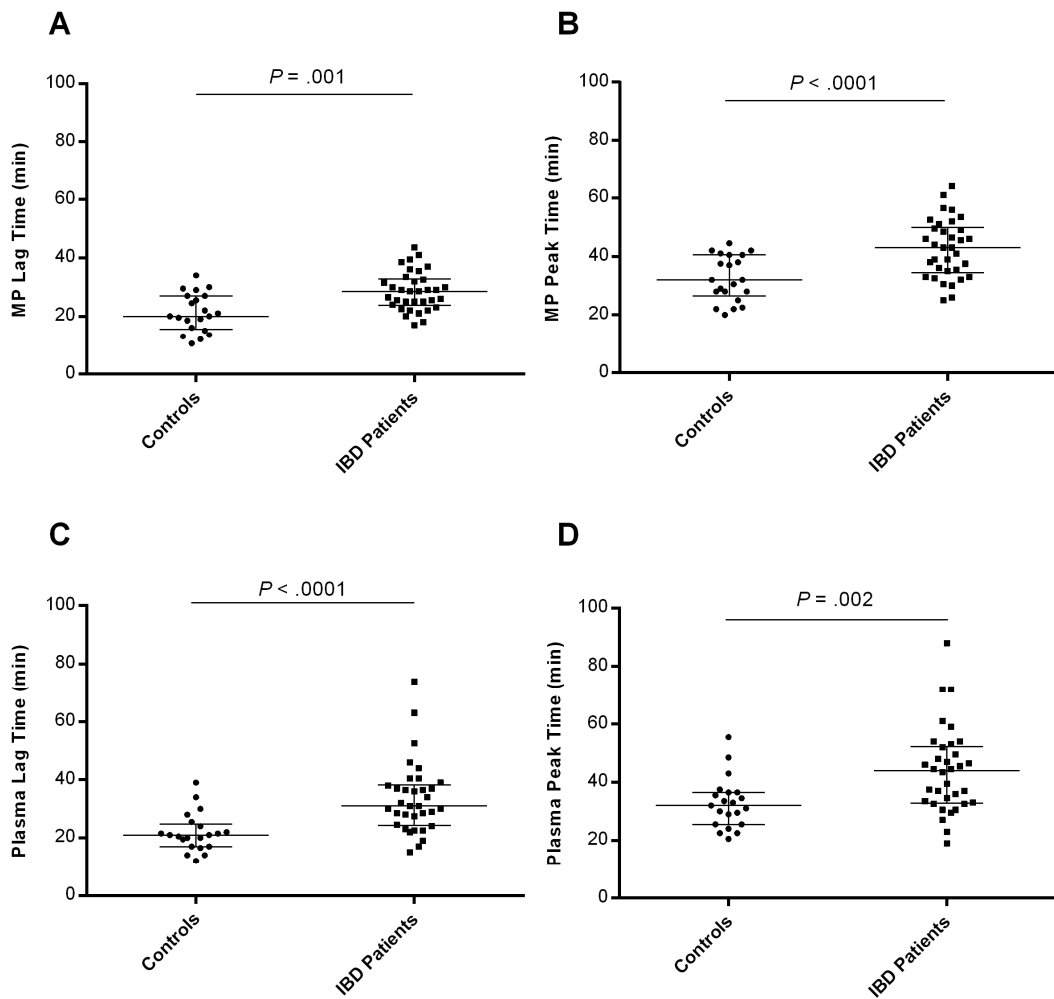
Figure 7.5: Comparing MP-mediated and whole plasma lag time and peak time between paediatric IBD patients and healthy controls

*MPs isolated by centrifugation were resuspended in pooled healthy MP-depleted plasma, and assessed along with platelet poor plasma (PPP) for thrombin generation kinetics by a fluorogenic thrombin generation assay (Pathway Diagnostics). IBD patients showed increased MP-mediated thrombin production lag time **(A)** and time to peak thrombin production **(B)**. In addition, IBD patients showed increased whole plasma thrombin production lag time **(C)** and time to peak thrombin production **(D)**. Results shown as median with inter-quartile range. Differences between controls and patients were assessed with Mann-Whitney U test.*

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7.3.3 Tissue Factor (TF) and TF Pathway Inhibitor (TFPI) activity in paediatric IBD

Both the standard curves obtained for TF (Figure 7.6A) and TFPI (Figure 7.6B) were as suggested by the manufacturers, with TF showing a positive sigmoidal relationship, and TFPI showing a negative sigmoidal relationship.

When comparing circulating TF activity in paediatric IBD patients with healthy adolescent controls, IBD patients showed a statistically significant (P = 0.0278) increase in TF activity (Figure 7.7A). While TFPI activity in paediatric IBD also showed a trend to increase compared to controls, it was not significant (P = 0.1047), most likely due to the high observed variability in IBD patients (Figure 7.7B). When comparing the TF:TFPI activity ratio, there was no significant difference (P = 0.5065) between paediatric IBD and controls (Figure 7.7C), but a relationship was seen when represented as a scatter plot of TF activity vs TFPI activity (Figure 7.7D). Healthy controls showed an inverse linear relationship between TF and TFPI activity (R^2 = 0.23, P = 0.026), but paediatric IBD patients showed no significant linear relationship between TF and TFPI activity (R^2 = 0.01, P = 0.505). This data suggests an intimate coupling of TF and TFPI in health, a relationship that appears to become uncoupled in IBD.
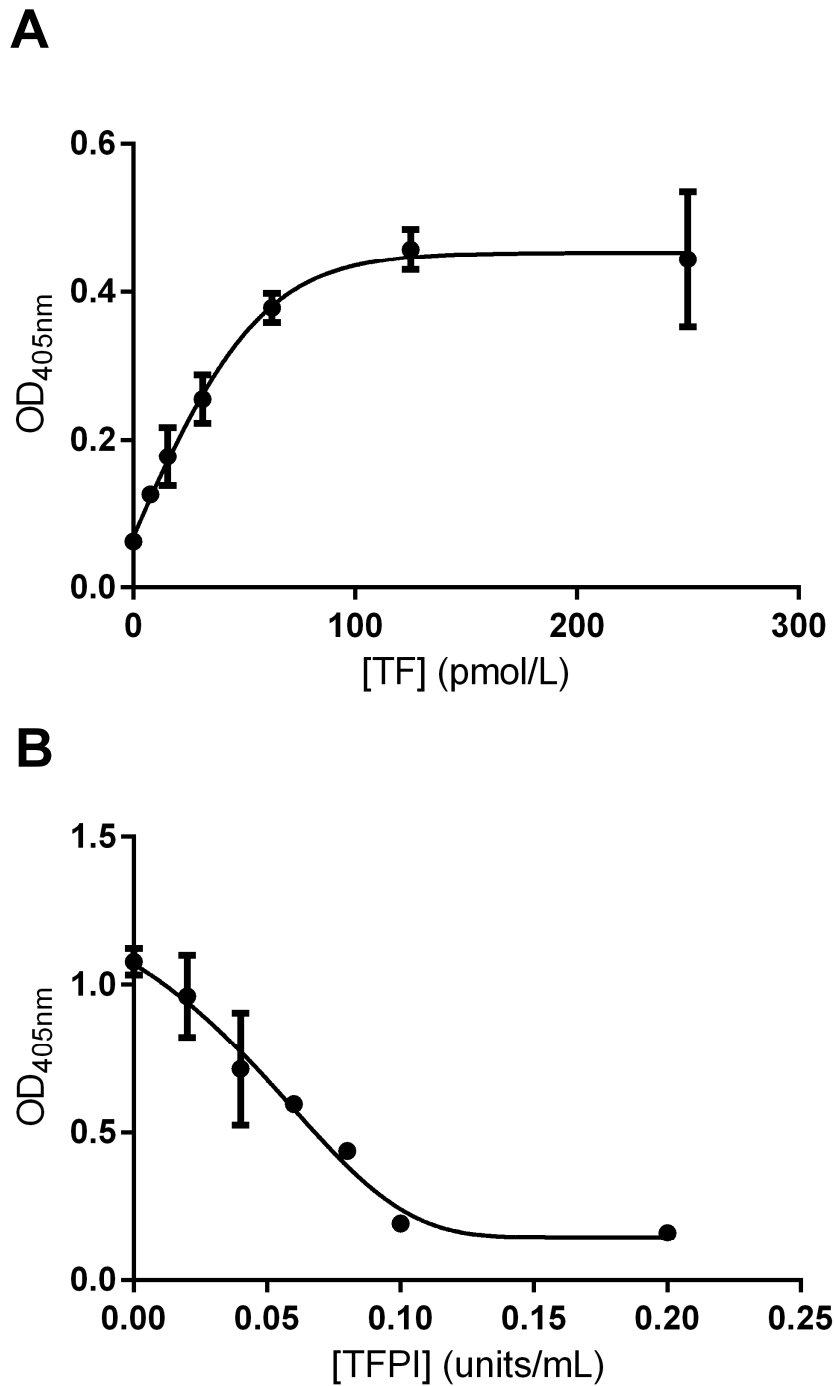
Figure 7.6: Standard curves of Tissue Factor (TF) and Tissue Factor Pathway Inhibitor (TFPI) activity assays

Commercial chromogenic TF (Abcam, UK) and TFPI (Sekisui Diagnostics, USA) activity assays were run as per kit instructions, with standards serially diluted in provided assay diluent. Curves were fitted by asymmetric 5-parameter logistic regression, with the TF activity standard curve **(A)** showing a positive relationship between optical density and TF activity, the TFPI activity standard curve **(B)** showing a negative relationship between optical density and TFPI activity.
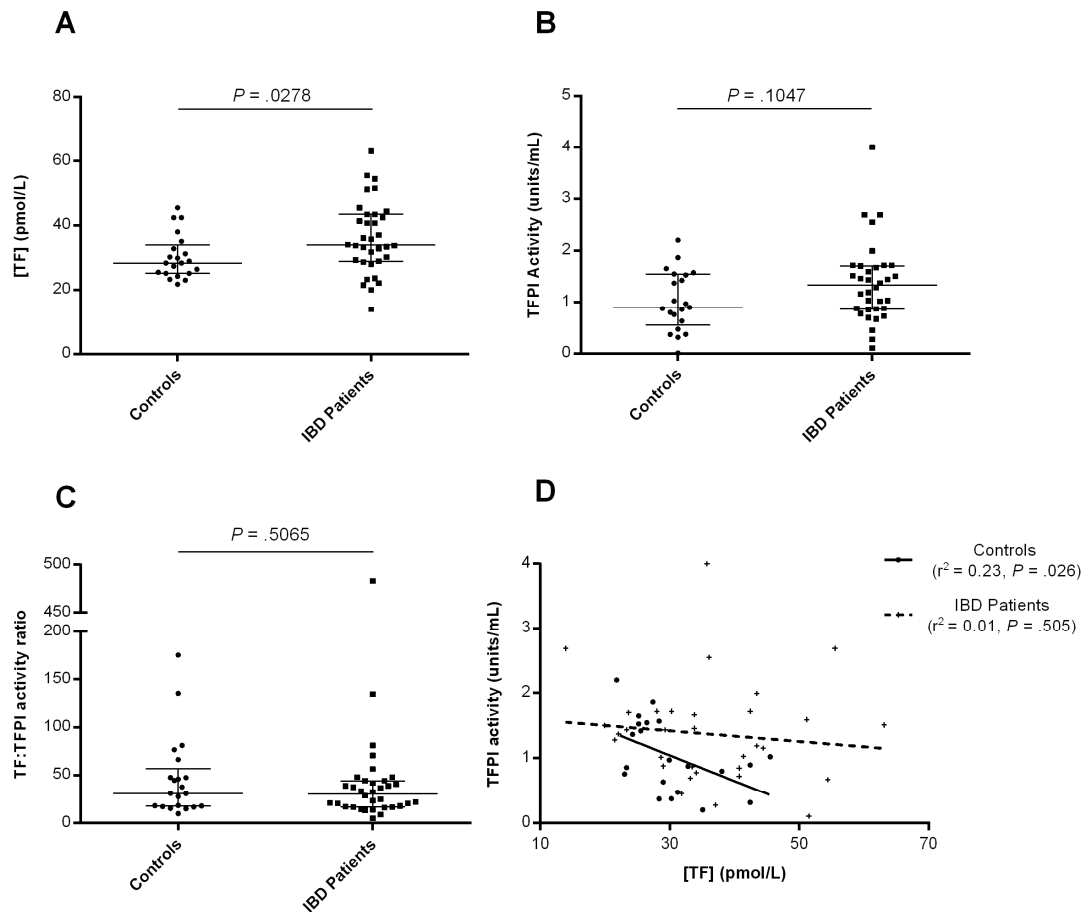
Figure 7.7: Tissue Factor (TF)/Tissue Factor Pathway Inhibitor (TFPI) activity axis is uncoupled in paediatric IBD compared to healthy adolescent controls.

(A) TF activity was assessed in platelet poor plasma (PPP) by measuring the ability of lipoprotein TF/FVIIa to activate FX to FXa utilising a commercial chromogenic kit (Abcam). Paediatric IBD patients (n = 34) showed a significant (P = .0278) increase in TF activity compared to adolescent controls (n = 21). (B) TFPI activity was assessed in PPP by measuring the ability of TFPI to inhibit the activity of exogenous TF/FVIIa complex to activate FX to FXa, by a commercial chromogenic kit (Sekisui Diagnostics). Paediatric IBD patients showed no significant (P = .1047) increase in TFPI activity, though median TFPI activity was increased (0.89 vs 1.33 units/mL). (C) TF:TFPI ratio was assessed and no difference (P = .5065) was observed between paediatric IBD patients and adolescent controls. All data shown as median with interquartile range, and differences were assessed by Mann-Whitney U test. (D) Correlation between TF and TFPI activity was assessed by linear regression, with healthy controls showing significant negative

correlation (r^2 = 0.23, P = .026); in contrast paediatric IBD showed no significant correlation (r^2 = 0.01, P =.505).

7.4 Discussion

IBD patients in the present cohort showed ongoing vascular endothelial injury (Chapter 5) and increases in various MP subtypes, including TF-expressing MPs (Chapter 6), hinting at the potential for paediatric IBD patients to be in a prothrombotic state. The goal of this study was to try to find evidence of this, and to ascertain if this state was MP-mediated.

The initial sample transport experiment was done to ascertain if previously described effects of cold on activating factor VII and factor VIII (Engbers et al., 2012) would materially affect thrombin generation parameters in the timeframe of sample collection (1h). Ideally samples would be kept on ice before processing into platelet poor plasma to prevent further release of MPs (Shah et al., 2008), so determining the ideal sample transport conditions to prevent biases in both assays was required. Figure 7.3-4 show that storage of blood on ice for 1h had no significant effect on any thrombin generation parameter measured, whether the samples were stimulated to release MPs using the calcium ionophore A23187 or not. This lack of significant change in thrombin generation parameters was maintained when looking at MP-mediated or whole plasma thrombin generation. The fact that there were trends to a prothrombotic state, with reduction in lag and peak time, and endogenous thrombin potential, peak thrombin and velocity index increasing upon incubation with A23187, demonstrated the sensitivity of the thrombin generation assay to increased levels of MPs, and gave confidence in the protocols used to assess thrombin generation in paediatric IBD patients.

Contrary to expectations, and also to previous results found in childhood arterial ischemic stroke (Eleftheriou et al., 2012) and paediatric IBD (Deutschmann et al., 2013), there was no observed increase in endogenous thrombin potential and no change in velocity index or peak thrombin when comparing paediatric IBD patients to controls, whether from whole plasma or MP-mediated thrombin generation (Table 7.1). Instead, there was a statistically significant increase in both lag time and time to peak thrombin, both in whole plasma and MP-mediated thrombin generation (Figure 7.5). This suggested that while the overall thrombin produced

was the same in IBD patients, with same velocity of production, there was a delay in this production compared to healthy controls. Additionally, the fact that these increases were similar in plasma and isolated MPs suggests the majority of this effect was being mediated by the MPs. While previous results have shown an increase in TF-expressing MPs (Chapter 6), the opposite expected effect is being seen in the thrombin generation kinetics of IBD patients. This is also contrary to the findings of previous studies, though there are some potentially mitigating factors. When looking at childhood arterial stroke, Eleftheriou *et al* used the same protocol for their thrombin generation assay, but collected and transported samples at room temperature to prevent activation of FVII and FVIII. We have shown in stimulated and unstimulated healthy volunteer blood that transport on ice doesn't change observed thrombin generation parameters (Figure 7.3-4), so this is unlikely to explain the difference in findings. It could be that a compensatory mechanism of some kind is present in paediatric IBD counteracting the increase in circulating TF. One obvious candidate for this is TFPI (Peraramelli et al., 2014), which is discussed below. While Deutschmann et al looked at thrombogenic potential in paediatric IBD, they used phospholipids and TF as reaction triggers to initiate their thrombin generation assays, which could mask the effect of phospholipids and TF from the samples themselves. It is for this reason we did not use a reaction trigger in our thrombin generation assays. However, this discrepancy in protocols is unlikely to explain the observed increases in lag and peak time observed in our IBD patients.

In order to further investigate these unexpected results, the overall plasma activities of TF and its main inhibitor, TFPI (Peraramelli et al., 2014), were investigated by commercial chromogenic assay. While TF activity was raised as expected (Figure 7.7A), TFPI activity was also raised, but not significantly (Figure 7.7B). Since these two proteins act in direct antagonism of each other, the ratio of activity of the two was investigated (Figure 7.7C), though there were no differences in ratio between controls and paediatric IBD. To further probe the relationship between these two proteins, each subject had their activities plotted on

a scatter plot. When only controls were looked at, there was a clear inverse linear relationship between TF and TFPI activity (Figure 7.7D). This could reflect the spectrum of "thrombogenicity" in healthy adolescents, with those showing higher TF activity having corresponding lower TFPI activity to promote coagulation, and vice-versa. Most interestingly however, was that no linear relationship whatsoever was observed between TF and TFPI activity in paediatric IBD. An easily observable relationship in healthy adolescents was not present in IBD, suggesting a compensatory process in health had somehow become decoupled in disease. This phenomenon should be studied in a larger cohort to try and ascertain if there is a predictive relationship between TF/TFPI activity and thrombin generation parameters. An important additional feature of a larger cohort would be to increase confidence in the observed increases in thrombin generation lag time and peak time, as this disagrees with the limited published evidence that is currently available.

8. Evidence for Structural Arterial Disease in Paediatric IBD

8.1 Introduction

Previous findings in our study cohort have demonstrated that paediatric IBD patients show evidence of endothelial injury (Chapter 5), increased circulating MPs (Chapter 6) and altered thrombin generation and Tissue Factor/Tissue Factor Pathway Inhibitor activities (Chapter 7). These initial changes to the vasculature are part of a potential progression to atherosclerosis, via vascular remodelling (Papafaklis et al., 2010). These subsequent changes can be classified as structural arterial disease, as the mechanical structure of the vasculature is being remodelled and stiffened.

Measuring these changes in vascular stiffness non-invasively is primarily achieved using oscillometric cuffs (Müller et al., 2013; Ring et al., 2014). As the ventricles of the heart contract during systole, a pulse wave is sent through the vasculature and down the artery walls. The speed that this pulse wave travels is proportional to the elasticity of the vessel wall. In order to measure this pulse wave, oscillometric cuffs are inflated around two arteries, one proximal and one distal to the heart. As the systolic pulse wave travels past the cuff, the momentary change in pressure, measured in fractions of a mmHg, is passed onto the cuff and recorded by the Vicorder instrument (Pucci et al., 2013). The time delay in measuring this pressure change is a function of the speed of the pulse wave and the distance the wave has had to travel. Measuring this distance for carotid-femoral pulse wave velocity (PWV) is the largest source of error in the measurement, and different methods exist to estimate the path length of the wave (Girerd et al., 2012). The most common and easily reproducible method for estimating path length is to measure from the carotid cuff to the suprasternal notch, and then from the suprasternal notch to the centre of the femoral cuff, summing these two values together. Some have investigated the effect of subtracting the length of the carotid artery (distance from suprasternal notch to carotid cuff) from the measurement to take into account the path

two separate hypothetical waves take: from the heart to the carotid cuff and from the heart to the femoral cuff. While producing substantially different PWV measurements, all of the investigated measurements still significantly correlated with each other (Sugawara et al., 2010). Paediatric reference ranges have been established, but by using a distance value of 80% of the direct distance between both cuffs (Fischer et al., 2012). This highlights the importance of stating the distance measurement used when measuring PWV. While carotid-femoral is the most well studied PWV measurement, other measurements such as carotid-radial (Liu et al., 2014) and brachial-ankle (E. K. Kim et al., 2015) PWV have been used to identify vascular changes. Carotid-femoral PWV is used because it has been well studied, covers a long path of large vessel walls, and is shown to positively correlate with age as the vasculature stiffens (Kozakova et al., 2015).

Carotid-femoral PWV has been measured multiple times in adult IBD, and a meta-analysis of these studies showed it to be increased in IBD, with the addition of anti-TNF-α treatment associated with a reduction in PWV (Zanoli et al., 2016). However, no work has been done on the paediatric population to show if PWV starts to increase early on in disease progression. Demonstrating changes in PWV in children with IBD would not only minimise many potential confounding factors such as diet, activity levels, alcohol intake and smoking, but could also provide an early warning sign and evidence of ongoing atherosclerotic changes that could be managed early in life to help prevent problems later on.

8.2 Methods

8.2.1 Study Participants

The participants used for this study are described in detail in Chapter 4. Briefly, 35 paediatric

IBD patients were recruited [median age 13.7y (7.8-16.5), 63% males, median BMI 21.8 (16.2,

27.0)]: 20 CD, 8 UC and 7 IBD-U. All were classified by Physicians Global Assessment score as

inactive (quiescent, n=27) or active (mild, n=8) IBD. 21 adolescent controls were recruited

[median age 17.0y (12.6, 18.4), 33% males, median BMI 19.1 (14.0, 35.7)], and 34 age and sex

matched control data were selected from a previous study [median age 12.2y (7.7, 17.7), 59%

males, median BMI 19.9 (13.5, 31.3)] (Shah et al., 2015).

8.2.2 Carotid-Femoral Pulse Wave Velocity (PWV)

Carotid-femoral PWV was measured by Vicorder instrument (Skidmore Medical, UK) as per

Chapter 2.2.8.

8.3   Results

8.3.1   Assessment of healthy controls

Since PWV is known to correlate strongly with age (Kozakova et al., 2015), subjects were first assessed with regards to this correlation. The healthy control subjects recruited for this study were adolescents and had higher median age than the IBD patients recruited (Chapter 4, $P <$ 0.0001). While IBD patients showed a positive linear correlation between PWV and age (Figure 8.1A, $R^2 = 0.22$), the healthy controls showed no correlation ($R^2 = 0.01$) and were far older than the IBD patients. When comparing the same IBD patients with age and sex-matched healthy controls from a previous study (Shah et al., 2015), a positive linear correlation between age and PWV was seen with IBD patients and historic controls (Figure 8.1B). The manner of correlation between these two variables was such that they could be described for IBD patients and historic controls with one linear regression line ($R^2 = 0.30$). For this reason, further analysis of carotid-femoral PWV was assessed using the recruited IBD patients and the age and sex-matched historic controls.
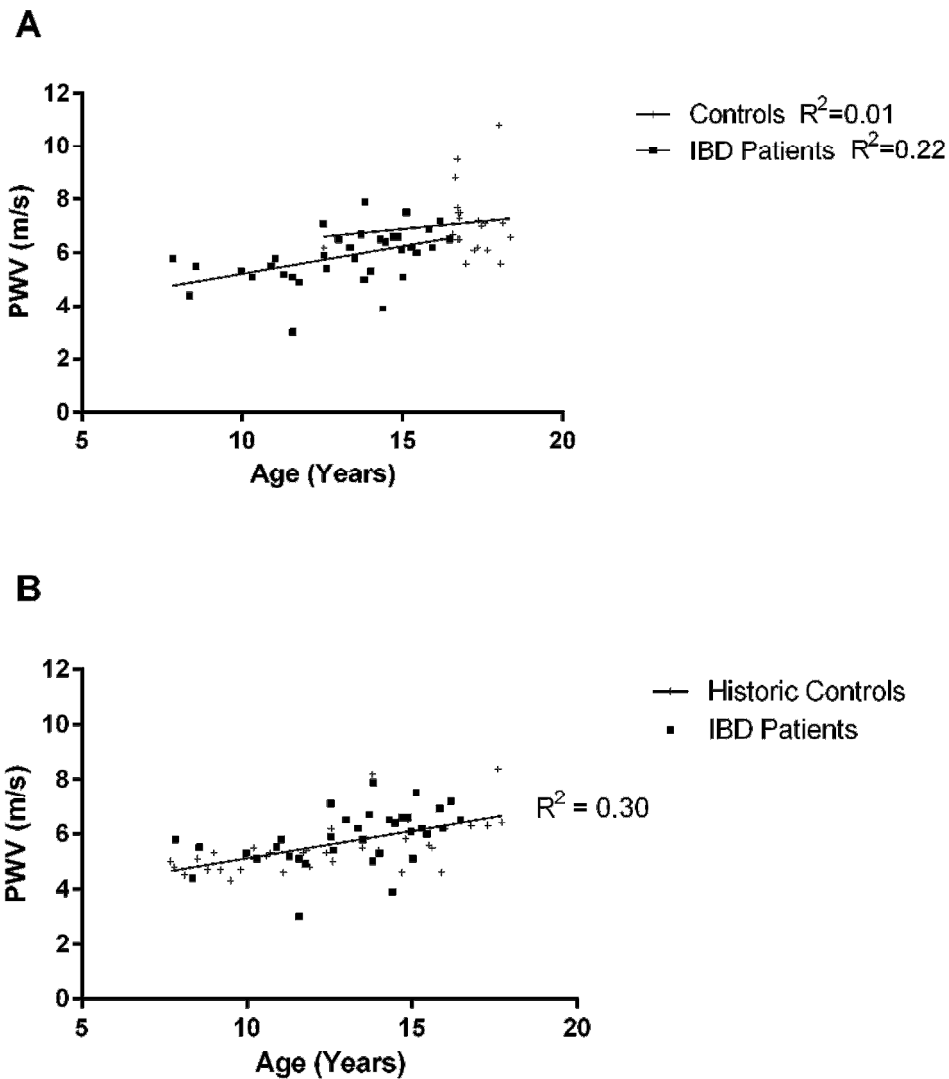
Figure 8.1: Relationship between age and carotid-femoral pulse wave velocity (PWV) in paediatric IBD

patients and different healthy controls

Carotid-femoral PWV was assessed by Vicorder instrument (Skidmore Medical, UK) in paediatric IBD

patients and (A) healthy adolescent controls, and then correlated with age at investigation. IBD patients

showed a positive linear correlation (R^2 = 0.22) between PWV and age, while adolescent controls showed

no linear correlation (R^2 = 0.01) between PWV and age. (B) The same paediatric IBD patients were also

compared with age and sex-matched healthy controls from a previous study (Shah et al., 2015). A single

linear regression line described the correlation between age and PWV for IBD patients and controls (R^2 =

0.30). Correlations were assessed by least-squares linear regression.

8.3.2 Regression analysis for predictors of carotid-femoral PWV

Lipid levels for historic controls were taken in a fasted state, whereas lipids for those recruited for the current study were taken as non-fasting. Non-fasting lipids have been shown to be just as effective for determining cardiovascular risk as fasting lipids (Sidhu and Naugler, 2012), however this difference in sample acquisition was noted. Due to the observed discrepancies in median age between recruited controls and recruited IBD patients and observed strong correlation between PWV and age (Figure 8.1), historic controls were used for linear regression modelling regardless of the difference in lipid assessment.

To initially assess the predictive capability of potential confounding variables on PWV, each was individually assessed by unadjusted univariate linear regression modelling (Table 8.1). The only statistically significant predictors of PWV were age ($P < 0.0001$) and HDL ($P = 0.007$). Presence of inactive IBD ($P = 0.056$) was close to being a significant predictor.

Variable	Unadjusted increase in mean PWV (95% CI)	P value
Presence of IBD (IBD vs control)	0.377 (-0.082, 0.836)	**P = 0.106**
Age at Investigation (Years)	0.199 (0.126, 0.272)	**P < 0.0001**
Presence of Inactive IBD	0.457 (-0.011, 0.924)	**P = 0.056**
HDL (mmol/L)	-0.732 (-1.261, -0.203)	**P = 0.007**
Diastolic BP (mmHg)	0.003 (-0.029, 0.035)	P = 0.862
Hs-CRP (mg/L)	0.002 (-0.010, 0.014)	P = 0.791
Systolic BP (mmHg)	0.012 (-0.010, 0.034)	P = 0.274
Presence of Active IBD	-0.137 (-0.872, 0.597)	P = 0.711
Serum Amyloid A	0.001 (-0.006, 0.008)	P = 0.750
Males	0.375 (-0.094, 0.844)	P = 0.115
VLDL (mmol/L)	-0.002 (-0.742, 0.738)	P = 0.996
Triglycerides (mmol/L)	-0.033 (-0.389, 0.324)	P = 0.855
BMI (m^2/kg)	0.042 (-0.010, 0.095)	P = 0.110
LDL (mmol/L)	0.063 (-0.317, 0.443)	P = 0.741
Total Cholesterol (mmol/L)	0.215 (-0.545, 0.125)	P = 0.215

Table 8.1: Univariate Predictors of carotid-femoral Pulse Wave Velocity (PWV) using historic controls.

Unadjusted univariate analysis for increase in mean PWV (m/s) in association with presence of IBD, age at investigation, presence of inactive IBD, high density lipoprotein (HDL), diastolic blood pressure, high-sensitivity c-reactive protein (hs-CRP), systolic blood pressure, presence of active IBD, serum amyloid A, sex (male), very low density lipoprotein (VLDL), triglycerides, body mass index (BMI) z-score, low density lipoprotein (LDL), total cholesterol, duration of disease and age of diagnosis. Those with a significant P value are shown in bold.

To fully assess the predictive capabilities of confounding variables while taking into account all other variables, a multivariate linear regression model was created taking into account the variables in Table 8.1. All variables were entered into a multivariate linear regression model, and then a backwards algorithm was performed. Through each iteration of the algorithm, the least significantly predictive variable was removed that had a predictive significance of $P >$ 0.10. The overall change in R^2 of the model was also assessed to see if it had changed significantly from the previous iteration. The final model was found when either there were no

more variables to remove which had predictive significance of $P > 0.10$, or removing a variable significantly changed the overall R^2 predictive power of the model. The final model once these conditions had been satisfied was:

PWV(m/s) = 4.054 + 0.187 * Age(years) – 0.578 * HDL (mmol/L), R^2 = 0.068, $P < 0.0001$

To best predict the PWV of the historic controls and recruited IBD patients in the study, only age and HDL levels were taken into account, with each increase in age by one year increasing PWV on average by 0.187m/s, and each increase in HDL by one mmol/L decreasing PWV on average by 0.578m/s. While presence of inactive IBD was almost significantly correlated with PWV ($P = 0.056$) in univariate analysis, once age and HDL had been taken into account the presence of IBD had no significant influence on PWV.

8.4 Discussion

While it has been demonstrated that paediatric IBD patients show evidence of endothelial injury (Chapter 5), increased circulating MPs (Chapter 6) and altered thrombin generation and Tissue Factor/Tissue Factor Pathway Inhibitor activities (Chapter 7), no-one has demonstrated structural changes to the vasculature in paediatric IBD with PWV. The use of carotid-femoral PWV enabled a non-invasive measure of arterial stiffness that was quick, reproducible and performed on a portable machine (London et al., 2004). The addition of a widely accepted consensus protocol (Urbina et al., 2009) provides a robust framework to help produce reliable data.

Initial analysis of recruited controls, plotting age against PWV (Figure 8.1A) , showed that age correlates strongly with PWV as previously demonstrated (Kozakova et al., 2015). However, due to the recruited controls not overlapping in age with the recruited IBD patients, using the recruited controls would involve extrapolating beyond the age range of the data to make a comparison with the IBD patients. It was therefore decided to use historical data of controls from a previous study (Shah et al., 2015), enabling controls to be age and sex matched to the recruited IBD patients. When plotted against age, the PWV of the historic controls showed a correlation that could be described with the same regression line as that for IBD patients (Figure 8.1B). This suggests that the PWV of healthy controls is no different to the PWV of paediatric IBD patients. This was confirmed when looking at potential confounding factors, as when age and HDL had been accounted for in a multivariate linear regression, presence of IBD was not a significant predictive factor. It must be taken into account that the lipid values taken for the historic controls were fasted, whereas the lipids taken for the paediatric IBD patients were non-fasted. This means the correlation with HDL, while interesting, should be looked at with scepticism until confirmed with samples all taken in the same fasting state. The fact that increased HDL appears to decrease PWV and therefore vascular stiffness however, does fit

with the overall cardio-protective role that HDL appears to have in healthy individuals (Namiri-Kalantari et al., 2015).

While it has been shown that adult IBD patients have increased PWV compared to healthy controls (Zanoli et al., 2012), the lack of changes seen in paediatric IBD in this study suggests the effect on arterial stiffness of IBD could be chronic and cumulative. A study to investigate when this accelerated change in arterial stiffness occurs by following patients longitudinally would provide valuable information not only as to when these changes occur, but also to look at factors such as disease severity, duration, treatment etc. to assess which could be predictive of accelerated changes in arterial stiffness.

The main assumption of PWV, that vessels are free with incompressible fluid within them, gives a good physical basis for higher PWV meaning a stiffer artery, but it has recently been shown that if the vessel is tethered at all, i.e. held in an extracellular matrix, this assumption breaks down. Increased degrees of tethering actually reduce hypothetical PWV, making PWV more of a measure of the ratio of vessel wall stiffness to vessel wall tethering (Hodis and Zamir, 2011). This then changes the central assumption of PWV as a metric for arterial stiffness, as it will only be a true proxy for arterial stiffness if the degree of arterial tethering stays constant within and between individuals. Since this has not been demonstrated, PWV changes should be viewed with a degree of scepticism and should backed up with supporting evidence before it can be said with confidence that a person has atherosclerotic stiffening of the arteries.

While PWV has been used in this study as a metric of arterial stiffness, another sensitive non-invasive marker of atherosclerotic changes is the measurement of the carotid intima media thickness (cIMT). It has been demonstrated in paediatric IBD patients that while there is no significant increase in cIMT, they have increased aortic intima media thickness (Aloi et al., 2012), indicating early atherogenic changes. Interestingly however, it has also been shown in a recent meta-analysis that changes in intima media thickness are not actually predictive of

future cardiovascular events (Lorenz et al., 2012). Therefore, the use of cIMT measurements in future studies in IBD should be balanced against the higher cost of the instrument and more extensive training required to use the technique compared to PWV.

9. Circulating Cytokine Profile in Paediatric IBD

9.1 Introduction

Circulating cytokines are involved in numerous processes both anti and pro-inflammatory within the body, and as such have been studied quite extensively in a variety of inflammatory and autoimmune diseases (Siebert et al., 2015; Brennan et al., 1995). Combined with the observed synergistic effect of the proinflammatory cytokines TNF-α, IL-17A and IFN-γ on endothelial MP release as described in Chapter 3, assessing the circulating levels of cytokines in paediatric IBD can lead to a greater understanding of not only disease pathogenesis, but may also shed light on therapeutic targets. This is confirmed by the current efficacious use of anti-cytokine biologics in the mainstream treatment of IBD.

One cytokine that has been studied well in IBD is tumour necrosis factor (TNF)-α (Jones-Hall and Nakatsu, 2016). This pro-inflammatory cytokine acts on many cell types including those of the innate immune system and the endothelium via specific TNF receptors, leading to cellular activation, proliferation and at high doses apoptosis via activation of NF-κB (Li and Lin, 2008; Li et al., 2014). Anti-TNF-α antibodies have been developed and shown to be effective at treating some IBD patients refractive to other treatments, first with the human-mouse chimeric monoclonal infliximab (Costa et al., 2013), and more recently the fully-humanised monoclonal antibody adalimumab (Roblin et al., 2014). The partial success of anti-TNF therapies has led to the investigation of other cytokine targets in IBD, some of which are further expanded on in the discussion.

Circulating cytokines have also been investigated as possible biomarkers for disease in IBD, though with less success. The EMerging BiomARKers in Inflammatory Bowel Disease (EMBARK) study looked at a variety of faecal and serum cytokines as potential biomarkers in CD and UC, and found that the combination of faecal calprotectin and matrix metalloprotease (MMP) 9 in CD, and faecal calprotectin, MPP9 and interleukin (IL)-22 in UC correlated with disease severity

(Faubion et al., 2013). However these biomarkers were not particularly sensitive and still had wide ranges of values in patients with the same disease activity. Faecal calprotectin is routinely measured in the clinic to monitor activity of IBD, but is used in conjunction with other clinical and histological assessments to inform treatment. Its high sensitivity to disease means a negative result can be used to rule out IBD with confidence, but a meta-analysis has shown there is a risk of false positives (Waugh et al., 2013).

With the advent of sensitive multiplex protein assays such as the MesoScale Discovery (MSD) electrochemiluminescence assay (Chowdhury et al., 2009), it is easier than ever to accurately assess the level of multiple cytokines in a small volume blood sample. The aim of this investigation was to assess 23 different cytokines and vascular injury markers using commercial multiplex MSD assays to see if paediatric IBD patients demonstrate differences in their circulating cytokines and vascular injury markers compared to healthy controls. The hypothesis was that the previously shown markers of vascular injury in this thesis in children with inflammatory bowel disease would correlate with raised inflammatory and soluble vascular injury markers, not only giving potentially a biomarker to monitor progress of these changes, but also possibly give insight in to the mechanisms causing this injury.

9.2 Methods

9.2.1 Study Participants and Sample Collection

The participants used for this study are described in detail in Chapter 4. Briefly, 35 paediatric

IBD patients were recruited [median age 13.7y (7.8-16.5), 63% males, median BMI 21.8 (16.2,

27.0)]: 20 CD, 8 UC and 7 IBD-U. All were classified by Physicians Global Assessment score as

inactive (quiescent, n=27) or active (mild, n=8) IBD. 21 adolescent controls were also recruited

[median age 17.0y (12.6, 18.4), 33% males, median BMI 19.1 (14.0, 35.7)].

9.2.2 MesoScale Discovery (MSD) Electrochemiluminescent Assay

Immune mediators implicated in IBD pathogenesis were quantified by multiplex electro-

chemiluminescence assay (MesoScale Discovery, USA). These included:

granulocyte/macrophage colony stimulating factor (GM-CSF), interferon (IFN)-γ, interleukins

(IL)-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-15, IL-16, IL-17A, tumour

necrosis factor (TNF)-α, and TNF-β. Vascular biomarkers measured were: soluble intracellular

adhesion molecule (sICAM)-1, soluble vascular adhesion molecule (sVCAM)-1 and vascular

endothelial growth factor (VEGF). In practice, 10μL of PPP was incubated in multi-spot well

microplates with up to 10 different capture antibodies, each coated onto its own individual

electrode. Wells were washed and incubated with a secondary antibody, conjugated to a Sulfo-

Tag® molecule that fluoresces when a current is passed through it, all as per manufacturer's

instructions. Fluorescence was measured on the SECTOR imager 6000 instrument (MesoScale

Discovery, USA) and compared to known standards provided with the assay.

9.3 Results

9.3.1 Sensitivity of MesoScale Discovery (MSD) assays

In order to determine the sensitivity of the MSD assays to each cytokine analysed, standard curves produced for each cytokine were compared to the values obtained by the participant samples (Figure 9.1). The lower limit of detection (LLoD) was calculated as 2.5 x standard deviation (SD) above the bottom standard. The lower limit of quantification (LLoQ) was provided by the kit manufacturer (MesoScale Discovery) and taken as the lowest amount of cytokine that shows 80-120% spike recovery and coefficient of variation (CV) of <20%, according to their internal experiments. LLoD and LLoQ values are detailed in Table 9.1, along with the number of participant samples that were above these values. No samples measured were above the range of the standard curve for any cytokine. All cytokines showed at least 12 samples that were above the LLoD, except for IL-1β which had no samples above the LLoD, and IL-13 which had 3 samples above the LLoD with none of them over the LLoQ. These two cytokines were excluded from further analysis due to insufficient assay sensitivity. All samples with cytokine values below the LLoD had these values replaced with 0.5 x LLoD.
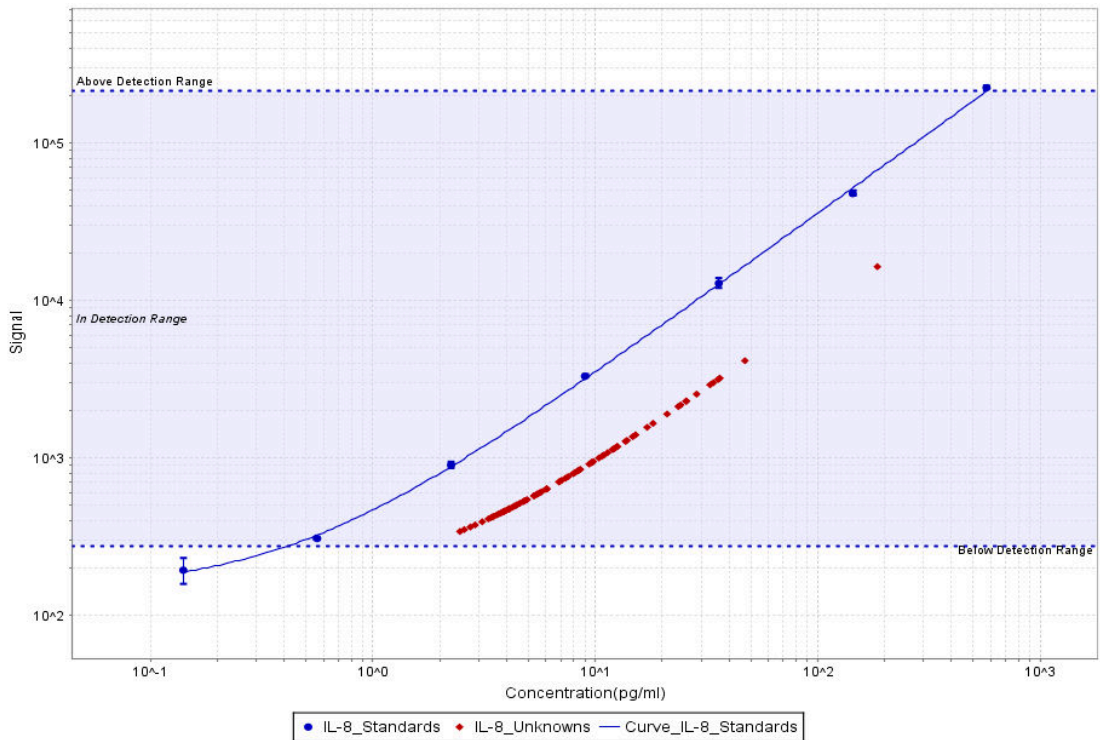
Figure 9.1: Example standard curve (IL-8) from MesoScale Discovery (MSD) assay of plasma from

healthy adolescent controls and paediatric IBD patients

Recombinant standard cytokines were reconstituted and diluted as per kit protocol, and run concurrently

with participant samples in duplicate. The resulting standard curve was produced by built-in software on

the SECTOR imager 6000 instrument (MesoScale Discovery, USA) by 5-parameter logistic regression, and

used to derive sample concentrations of each analyte (in this example, IL-8) from the sample

fluorescence value. Standard results are shown in blue, sample results from 35 paediatric IBD patients

and 21 healthy adolescent controls shown in red. The lower limit of detection (LLoD) was calculated as a

value 2.5 SD above the bottom standard value.

Cytokine	LLoD (pg/mL)	LLoQ (pg/mL)	Samples above LLoQ	Samples above LLoD
GM-CSF	0.77	1.90	5/56	15/56
IL-1α	0.27	2.85	49/56	55/56
IL-5	0.80	6.28	4/56	12/56
IL-7	0.45	1.37	52/56	52/56
IL-12p40	1.72	5.68	54/56	54/56
IL-15	0.36	1.40	54/56	54/56
IL-16	1.86	19.1	54/56	54/56
IL-17A	2.13	9.32	30/56	54/56
TNF-β	0.28	1.15	17/56	41/56
VEGF	1.19	7.70	55/56	56/56
IFN-γ	1.41	7.47	14/56	39/56
IL-1β	0.45	2.14	0/56	0/56
IL-2	0.46	0.890	5/56	21/56
IL-4	0.15	0.450	1/56	15/56
IL-6	0.31	1.58	10/56	43/56
IL-8	0.42	1.13	57/56	56/56
IL-10	0.26	0.680	29/56	54/56
IL-12p70	0.31	1.22	4/56	28/56
IL-13	0.78	4.21	0/56	3/56
TNF-α	0.24	0.690	56/56	56/56
CRP	30.61	54.0	56/56	56/56
SAA	196.3	27.6	56/56	56/56
sVCAM-1	14.69	37.6	56/56	56/56
sICAM-1	5.89	6.40	56/56	56/56

Table 9.1: Paediatric IBD patient and healthy adolescent control samples above the lower limit of detection (LLoD) and lower limit of quantification (LLoQ) for cytokines measured by MesoScale Discovery (MSD) assay

35 paediatric IBD patients and 21 healthy adolescent controls were assessed for the above plasma cytokines by MesoScale Discovery (MSD) multiplex chemiluminescent assay. For each individual analyte, the lower limit of detection (LLoD) was calculated from the standard curve as 2.5 x standard deviations above the value of the bottom standard. The lower limit of quantification (LLoQ) was given by the manufacturer, and calculated as the value of cytokine at which 80-120% spike recovery occurred, with a coefficient of variation (CV) of <20%. Abbreviations are: granulocyte/macrophage colony stimulating factor (GM-CSF), interleukin (IL), tumour necrosis factor (TNF), vascular endothelial growth factor (VEGF),

interferon (IFN), C-reactive protein (CRP), serum amyloid A (SAA), soluble vascular cell adhesion molecule

(sVCAM), soluble intercellular adhesion molecule (sICAM).

9.3.2 Comparing circulating cytokine levels of paediatric IBD patients and adolescent controls

When comparing the median circulating cytokine levels between paediatric IBD patients and recruited adolescent controls (Table 9.2), IL-12p40 (P = 0.044, 1.23-120.59 pg/mL), IL-15 (P = 0.035, 0.11-2.43 pg/mL), IL-17A (P < 0.0001, 2.34-10.91 pg/mL), IL-2 (P < 0.0001, 0.00-0.39 pg/mL), IL-6 (P = 0.010, 0.08-0.86), IL-8 (P < 0.0001, 1.35-6.04 pg/mL), IL-10 (P = 0.047, 0.00-0.489 pg/mL), TNF-α (P = 0.001, 0.66-2.37 pg/mL), sVCAM-1 (P = 0.002, 78,496-358,537 pg/mL) and sICAM-1 (P = 0.005, 66,752-348,225) all showed significant increases in paediatric IBD. These significant increases are shown in Figure 9.2.

Cytokine (pg/mL)	Mann-Whitney U P-value	Median difference from control (95% CI)
GM-CSF	P = 0.053	0.000 [0.000-0.298]
IL-1α	P = 0.438	1.892 [-2.884-7.474]
IL-5	P = 0.848	0.000 [0.000-0.000]
IL-7	P = 0.856	-0.274 [-2.810-1.934]
IL-12p40	**P = 0.044**	**56.75 [1.23-120.59]**
IL-15	**P = 0.035**	**1.11 [0.11-2.43]**
IL-16	P = 0.897	1.842 [-26.647-29.640]
IL-17A	**P < 0.0001**	**6.07 [2.34-10.91]**
TNF-β	P = 0.993	0.000 [-0.373-0.365]
VEGF	P = 0.742	9.626 [-56.203-72.923]
IFN-γ	P = 0.057	-1.358 [-4.474-0.000]
IL-2	**P < 0.0001**	**0.26 [0.00-0.39]**
IL-4	P = 0.270	0.000 [0.000-0.000]
IL-6	**P = 0.010**	**0.519 [0.08-0.86]**
IL-8	**P < 0.0001**	**2.79 [1.35-6.04]**
IL-10	**P = 0.047**	**0.22 [0.00-0.489]**
IL-12p70	P = 0.698	0.000 [0.000-0.230]
TNF-α	**P = 0.001**	**1.43 [0.66-2.37]**
CRP	P = 0.086	-385,641 [-1,919,355-51,783]
SAA	P = 0.729	-169,442 [-1,663,135-1,407,220]
sVCAM-1	**P = 0.002**	**200,031 [78,496-358,537]**
sICAM-1	**P = 0.005**	**203,411 [66,752-348,225]**

Table 9.2: Comparison of circulating cytokines between paediatric IBD patients and healthy adolescent controls by MesoScale Discovery (MSD) assay

35 paediatric IBD patients and 21 healthy adolescent controls were assessed for the above plasma cytokines by MesoScale Discovery (MSD) multiplex chemiluminescent assay. Group medians were compared by Mann-Whitney U test, with 95% confidence intervals calculated using Hodges-Lehman estimate. Abbreviations are: granulocyte/macrophage colony stimulating factor (GM-CSF), interleukin (IL), tumour necrosis factor (TNF), vascular endothelial growth factor (VEGF), interferon (IFN), C-reactive protein (CRP), serum amyloid A (SAA), soluble vascular cell adhesion molecule (sVCAM), soluble intercellular adhesion molecule (sICAM).
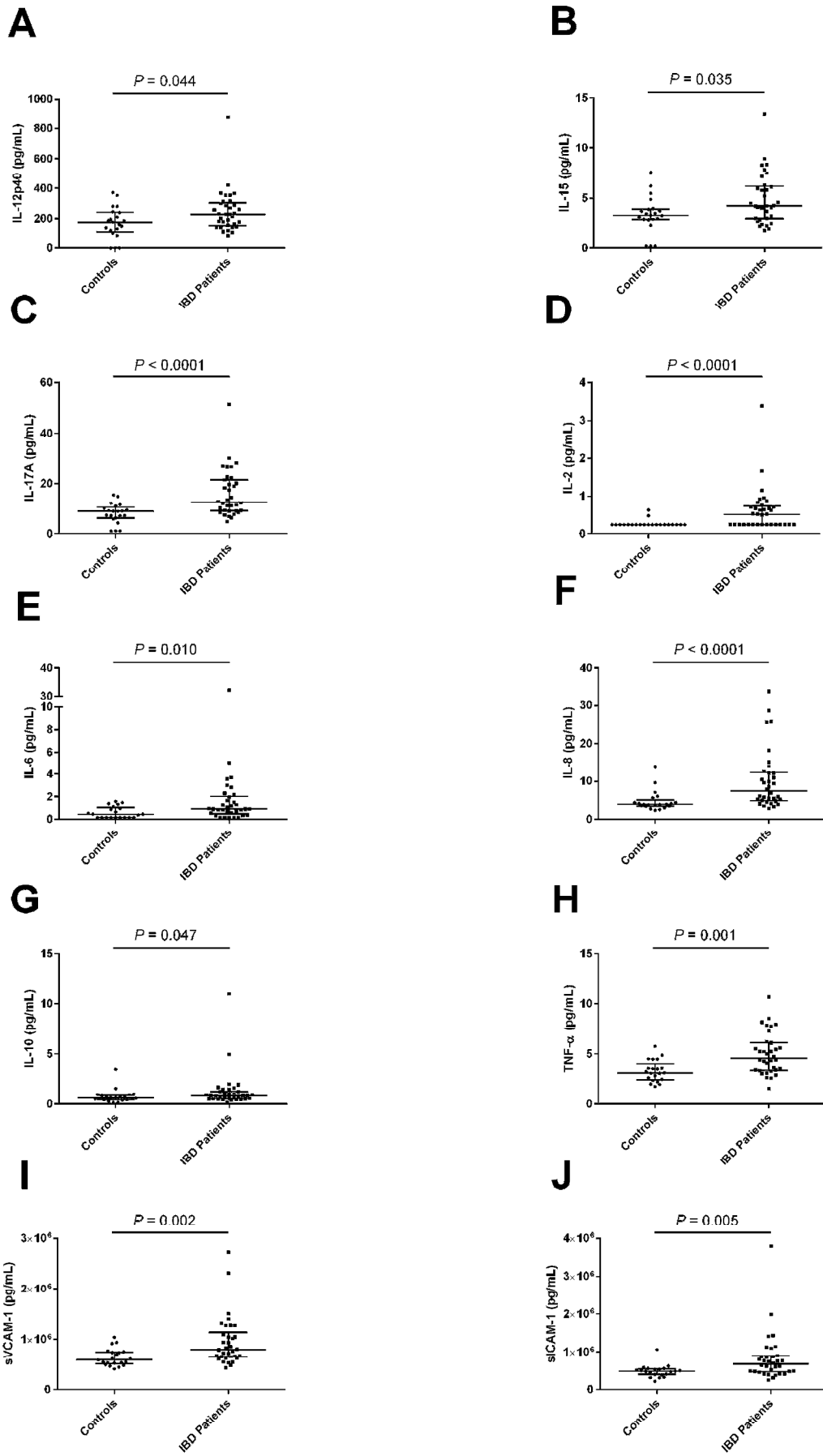
Figure 9.2: Comparison of circulating cytokines between paediatric IBD patients and healthy adolescent controls by MesoScale Discovery (MSD) assay

*35 paediatric IBD patients and 21 healthy adolescent controls were assessed for the above plasma cytokines by MesoScale Discovery (MSD) multiplex chemiluminescent assay. **(A)** Interleukin (IL)-12p40, **(B)** IL-15, **(C)** IL-17A, **(D)** IL-2, **(E)** IL-6, **(F)** IL-8, **(G)** IL-10, **(H)** tumour necrosis factor (TNF)-α, **(I)** soluble vascular cell adhesion molecule (sVCAM)-1 and **(J)** soluble intercellular adhesion molecule (sICAM)-1 all showed a statistically significant increase in paediatric IBD compared to controls. Group medians were compared by Mann-Whitney U test, with 95% confidence intervals calculated using Hodges-Lehman estimate.*

9.3.3 Regression analysis for predictors of circulating cytokines in paediatric IBD

To fully assess the predictive capabilities of confounding variables while taking into account all other variables, multivariate linear regression models were created for each cytokine taking into account presence of IBD, age at investigation, presence of inactive IBD, high-sensitivity c-reactive protein (hs-CRP), systolic blood pressure, presence of active IBD, serum amyloid A, sex (male), triglycerides, body mass index (BMI) z-score, total cholesterol, duration of disease and age of diagnosis. HDL, LDL, VLDL were all excluded from analysis due to significant collinearity with other variables, as established in Chapter 6. All variables were entered into a multivariate linear regression model for each cytokine, and then a backwards algorithm was performed. Through each iteration of the algorithm, the least significantly predictive variable was removed that had a predictive significance of $P > 0.10$. The overall change in R^2 of the models was also assessed to see if it had changed significantly from the previous iteration. The final models were found when either there were no more variables to remove which had predictive significance of $P > 0.10$, or removing a variable significantly changed the overall R^2 predictive power of the model. Final models for each cytokine are shown in Table 9.3.

Cytokine (pg/mL)	Overall model	R^2 of overall model	P value
GM-CSF	No model found	N/A	N/A
IL-1α	No model found	N/A	N/A
IL-5	No model found	N/A	N/A
IL-7	-2.137 + 0.066*(systolic BP) + 1.924*(Triglycerides)	0.123	P = 0.072
IL-12p40	172.443 + 75.580*(IBD)	0.079	P = 0.068
IL-15	-4.240 + 0.05*(systolic BP) + 1.955*(IBD) + 1.329*(triglycerides)	0.288	P = 0.004
IL-16	No model found	N/A	N/A
IL-17A	36.559 − 1.617*(age)	0.250	P = 0.001
TNF-β	0.222 + 0.790*(triglycerides)	0.116	P = 0.025
VEGF	No model found	N/A	N/A
IFN-γ	No model found	N/A	N/A
IL-2	0.258 + 0.347*(IBD)	0.116	P = 0.026
IL-4	-0.837 + 0.178*(male) + 0.225*(cholesterol)	0.201	P = 0.011
IL-6	-9.346 + 0.584*(age) + 3.895*(IBD)	0.103	P = 0.115
IL-8	4.695 + 5.465*(IBD)	0.152	P = 0.010
IL-10	No model found	N/A	N/A
IL-12p70	No model found	N/A	N/A
TNF-α	3.296 + 1.723*(IBD)	0.192	P = 0.003
CRP	No model found	N/A	N/A
SAA	No model found	N/A	N/A
sVCAM-1	1,791,718 − 65,875*(age)	0.175	P = 0.005
sICAM-1	1,729,049 − 71,093*(age)	0.129	P = 0.018

Table 9.3: Multivariate predictors of cytokine levels in paediatric IBD.

Multivariate analysis for mean increase in cytokine levels in association with presence of IBD, age at investigation, presence of inactive IBD, high-sensitivity c-reactive protein (hs-CRP), systolic blood pressure, presence of active IBD, serum amyloid A, sex (male), triglycerides, body mass index (BMI) z-score, total cholesterol, duration of disease and age of diagnosis. All above variables were entered into an initial multivariate regression, then the backwards algorithm with iterated with exclusion P value >0.10. Overall model with significant predictors, R^2 of overall model, and significance of overall model shown. Multivariate models that only include a constant in their final iteration are described as "no model found".

Of the cytokines that had previously been found to not be significantly changed in IBD, none of them had a multivariate linear regression model that included IBD as a predictor, confirming the lack of relationship between those cytokines and the presence of IBD. Of those cytokines previously found to be significantly increased in IBD, sVCAM-1, sICAM-1 and IL-17A had regression models that didn't include presence of IBD, and instead were negatively associated with age. IL-10 had no model found, suggesting a weak relationship between presence of IBD and IL-10.

9.4 Discussion

It has been shown in previous chapters that paediatric IBD patients demonstrate evidence of endothelial injury (Chapter 5), increased circulating levels of MPs (Chapter 6), and altered thrombin generation and Tissue Factor/Tissue Factor Pathway Inhibitor activities (Chapter 7). Combined with the evidence that proinflammatory cytokines provoke MP release in a synergistic manner (Chapter 3), it was pertinent to examine the circulating cytokine profile of these patients.

A variety of cytokines were found to be significantly increased in IBD (Table 9.2, Figure 9.2), though not all remained significant when taking into account other confounding factors (Table 9.3). IL-12p40, IL-15, IL-2, IL-6, IL-8 and TNF-α were confirmed to be increased in paediatric IBD by multivariate regression modelling, but the amount by which they were increased in all cases except IL-12p40 was in the region of 1-10pg/mL. The physiological relevance of such a small increase in circulating cytokines must be taken into account alongside the fact the increases are statistically significant. However, the fact that circulating levels of these cytokines only modestly increased does not preclude a greater increase inside the intestinal lumen where inflammation actually takes place in IBD (Hanauer, 2006).

An interesting feature was seen in the levels of IL-2, a potent mediator of anti-inflammatory T_{reg} proliferation (Malek, 2003). Rather than a change in relative levels of IL-2 between groups, all but 2 adolescent controls had IL-2 levels below the LLoD (Figure 9.2-D), whereas 20 of the 35 paediatric IBD patients had detectable levels. This either suggests IL-2 levels in plasma are right at the limit of detection of this assay, or there is a more binary relationship with this cytokine, with it being absent in health and present in IBD. A study in paediatric CD has shown increased levels of IL-2, which returned to healthy control levels when patients were treated with the anti-TNF-α biologic infliximab (Katz et al., 2014). It could be that this signal seen in IL-2 is a compensatory mechanism by the innate immune system attempting to dampen chronic inflammation by upregulation of T_{reg}s.

IL-15 is a functionally similar cytokine to IL-2, promoting T-cell and NK cell proliferation, and has been shown to be overexpressed in the lamina propria and mucosa-resident monocytes in IBD (Liu et al., 2000). The small increase in levels in circulation is likely to reflect this increase within the intestine. IL-6 is a relatively well-studied cytokine in IBD and thought to prevent the apoptosis of CD4+ T-cells by activating STAT3, thus prolonging inflammation (Mudter and Neurath, 2007). Anti-IL-6 treatments have been looked at, with the anti-IL-6 receptor antibody tocilizumab showing efficacy in treating rheumatoid arthritis and juvenile idiopathic arthritis (Kang et al., 2015). The demonstrated increases in circulating IL-6 in paediatric IBD patients suggest anti-IL-6 treatments would also be effective in this condition. IL-8 is also a well-studied cytokine shown to be increased in IBD. In the mucosa, IL-8 has been shown to be increased in IBD and correlate with active disease, and is thought to act by promoting the migration of neutrophils to the site of inflammation, and increasing their binding affinity for the extracellular matrix (Ina et al., 1997). Lamina propria IL-8 has also been shown to correlate with disease activity in paediatric IBD, but with no significant changes in circulating IL-8 (Reddy et al., 2007). In our cohort however, we have found a small but demonstrable increase in circulating IL-8 in paediatric IBD. TNF-α is probably the best-described cytokine involved with IBD pathogenesis, working by activating NF-κB and promoting cell activation, proliferation and at higher levels, apoptosis (Li and Lin, 2008; Li et al., 2014). The efficacy of anti-TNF-α antibodies infliximab and adalimumab in the treatment of IBD demonstrate its involvement in the disease (Thorlund et al., 2014). The increases of TNF-α seen in this paediatric cohort support that.

IL-12p40 was the cytokine in our cohort that demonstrated a marked increase in paediatric IBD compared to controls (Table 9.3). IL-12p40 is the common subunit of both IL-12 and IL-23, and polymorphisms of its gene, *IL12B*, have been associated with intestinal inflammation (Barrett et al., 2008). In addition to genetic associations, the anti-IL-12p40 antibody ustekinumab has been shown in phase 2b trials to be effective at inducing and maintaining remission in

refractive CD (Sandborn et al., 2012). The fact that paediatric IBD patients in the current study showed increased levels of IL-12p40 suggest ustekinumab may also be effective in treating paediatric IBD. It is also important to note that the majority of patients were in remission or had only mild symptoms, and yet IL-12p40 was substantially increased. The relationship between IL-12p40 and disease activity and severity warrants further attention.

VCAM-1, ICAM-1 and IL-17A originally showed an increase in paediatric IBD (Figure 9.2), but when assessed with multivariate linear regression modelling, none showed presence of IBD as a predictor (Table 9.3). However, they did all show a negative correlation with age. This may be a genuine correlation, but the fact that all healthy controls in this study were older than the IBD patients means we cannot be sure in this case. A further study investigating these parameters in properly age-matched controls is necessary to determine if these cytokines and vascular injury markers really are associated with paediatric IBD.

Overall, we have demonstrated statistically significant increases in various pro-inflammatory cytokines in paediatric IBD. However, the physiological relevance of the observed increases is hard to determine and requires further scrutiny. Many of the cytokines that have been shown to be increased already have a therapeutic antibody to them either in development, approved for use in another inflammatory disease, or already in use in IBD in the case of infliximab and adalimumab. Especially in light of the observed synergistic effect of proinflammatory cytokines on MP release demonstrated in Chapter 3, and the interdependent pathways that many of these cytokines act on, combination therapy with multiple biologics may bring about a greater degree of efficacy in the treatment of IBD. Conversely, the large interplay between different cytokine pathways mean extreme care must be taken to avoid unexpected side effects.

10. Discussion

IBD is a collection of life-long chronic relapsing and remitting inflammations of the GI tract, with significant cardiovascular morbidity (Singh et al., 2015). With incidence increasing, particularly within paediatric IBD (Lovasz et al., 2014; Martín-de-Carpi et al., 2014; Molodecky et al., 2012), these morbidities are also set to rise. While the mechanism of these cardiovascular events has been hypothesised to be due to premature atherosclerosis mediated by systemic inflammation (Singh et al., 2015), little work has been done in paediatric IBD to ascertain when these atherosclerotic changes occur in disease progression. Being able to detect changes to the vasculature early on in disease progression would give evidence to support earlier intervention, and provide biomarkers to monitor changes in the vasculature longitudinally. While limited individual studies have looked at the prothrombotic role of MPs in paediatric IBD (Deutschmann et al., 2013), indices of vascular injury, circulating MPs, vascular stiffness and the prothrombotic tendency of paediatric IBD has not been studied, let alone studied together in the same cohort. The overall purpose of this PhD was to investigate these indices in paediatric IBD, while attempting to elucidate the mechanisms involved in MP release.

10.1 Results Summary

Chapter 3 involved first optimising *in vitro* conditions for endothelial MP release and validating the detection and enumeration of MPs by flow cytometry. Once achieved, it was demonstrated that cytokines prototypically associated with IBD, namely TNF-α, IL-17A and IFN-γ, all stimulated MP release from primary human endothelial cells and an endothelial cell line. In addition to causing MP release alone, they acted synergistically when combined, causing significantly greater MP release in tandem with each other. Attempting to show that MP release could be inhibited by using small molecule inhibitors of ROCK-1, LIMK-2 and Src was inconclusive, with no consistent decreases in MP release seen with any inhibitors. However,

this was comparing MP production from unstimulated cells as a control, and if the control had been cytokine-stimulated cells to approximate an inflammatory disease patient, reductions could have potentially been seen. Autologous MP stimulation had a more pronounced effect, with MPs taken from TNF-α stimulated EA.hy926 cells increasing IL-8 production when incubated with unstimulated cells. The argument that this could be due to residual TNF-α is negated by a wash step, with potential contamination with cytokine being at least 10^6 times more dilute than the original concentration (50ng/mL). Similarly to the small molecule inhibitors, no decrease in MP release was seen in HUVECs or EA.hy926 cells when incubated with statins or methotrexate. This could again be due to the lack of initial stimulation with a pro-inflammatory cytokine, which could be assessed in future work. Obvious drawbacks to this *in vitro* approach are how non-physiological the conditions are, with a single cell type analysed in isolation with no laminar flow as in a blood vessel (discussed in Chapter 3), and the level of cytokines used to stimulate MP release orders of magnitude higher than those found *in vivo*. Isolated cellular models such as this have many drawbacks due to their highly simplified setting. This is useful for isolating factors involved but can often over-simplify the system investigated.

Chapter 4 summarised the clinical study conducted to look at the following questions:

- Do children with IBD show evidence of endothelial injury?

- Do children with IBD have evidence of a MP-mediated prothrombotic tendency?

- Do children with IBD show evidence of structural arterial disease?

- Do children with IBD show increased circulating pro-inflammatory or vascular injury markers?

To this end, 35 paediatric IBD patients were recruited from Great Ormond Street Hospital, along with 21 adolescent healthy controls, and data collected from 34 historic controls from a previous study (Shah et al., 2015), age and sex matched to the IBD patients. The historic controls had data collected on general clinical features, such as height, weight, and blood

pressure, as well as CECs and carotid-femoral PWV. Their lipid values, however, were assessed in fasting conditions unlike the recruited IBD patients which were assessed in a non-fasting state. Ideally the recruited controls should also have been age and sex matched to the IBD patients, but there was enormous difficulty in obtaining paediatric recruits for the study in the time-frame of this PhD. The fact that Great Ormond Street Hospital is a tertiary centre and admits complex patients referred from other hospitals minimises the likelihood of any fitting the criteria of a control for this study. A compromise position of recruiting adolescent controls who attended a study day at the Rayne Institute, UCL was found, with the proviso that particular attention would need to be paid to the potential confounder of age in subsequent analyses. This should be carefully considered when designing future studies, as the success of a clinical study relies heavily on how appropriate the control group is. Additionally, the large diversity of the patient group studied, with the wide range of presentations, current treatments and clinical histories make it difficult to draw clear conclusions. Despite these difficulties however, trends and differences between groups were still seen. Chapters 5-9 addressed the above questions using a variety of techniques.

Chapter 5 addressed the question of evidence for endothelial injury in paediatric IBD by investigating levels of CECs. These detached vascular endothelial cells are implicated in many aspects of vascular disease, and act as an early marker of atherosclerotic changes to the blood vessels (Cahill and Redmond, 2016). In the studied paediatric IBD patients, CECs were significantly raised compared to controls, and no other confounding variables, including age, were significantly correlated. This increase in CECs appeared independent of disease activity, with well-managed patients in remission also showing significant increases. While this means CECs appear an ineffective marker for disease activity stratification, this does mean that patients considered well-managed still have ongoing subclinical endothelial injury occurring. The fact only mildly active IBD patients were recruited to the study means the effect of more severe disease may show a further increase in CECs. Patients with severe disease were not

included in this thesis, however it is important to note that preliminary work from our laboratory suggests a further significant increase in CECs in severe disease. This highlights the role of CECs as a potential marker of vascular dysfunction in IBD and needs further clarification.

Chapter 6 evaluated the circulating MPs found in paediatric IBD by flow cytometry using a designed 7-marker panel. The 7-marker panel in this thesis is the most extensive panel available to study MP characteristics. After accounting for potential confounding variables, presence of IBD significantly increased the median circulating MPs overall, as well as neutrophil MPs, monocyte MPs, TF-expressing MPs, and monocyte MPs also expressing TF. All of these subsets had significant co-predictor variables when assessed by multivariate linear regression. All multivariate models included either total cholesterol or triglyceride levels as positive predictors of median MP levels, showing the importance of assessing lipid profile in patients when looking at circulating MPs. The increase in TF-expressing MPs does however show that paediatric IBD patients have increased circulating levels of a known pro-thrombotic factor (Mackman, 2004). The fact that the increased MPs seen in paediatric IBD appear to be derived from neutrophils, monocytes and endothelial cells highlights the effect of GI inflammation on multiple cell types, and that these MPs can be effectors of systemic inflammation. This is highlighted by a study that showed TF-expressing monocyte MPs can fuse with granulocytes which then express the TF on their surface, with no TF being produced by the granulocytes themselves (Egorina et al., 2008). Highlighting the increased levels of MPs in paediatric IBD is potentially just the beginning, with deeper phenotyping of MPs a possible route to follow, detailed later.

After establishing increases in paediatric IBD of a potential pro-thrombotic feature, Chapter 7 explored how MPs from these patients functionally affect thrombin generation, as well as functional activity of TF and its direct inhibitor TFPI. Results obtained from the thrombin generation assay were unexpected. While MP-mediated and whole plasma thrombin

generation showed the same endogenous thrombin potential, velocity of thrombin production, and peak thrombin production in paediatric IBD, patients showed an increase in lag and peak time of thrombin production compared to controls. This delay in thrombin production is contrary to the expected effect of IBD, which with increased circulating MPs and increased lifetime risk of thromboembolic complications, would be expected to show increased MP-mediated endogenous thrombin potential, as shown in a study on childhood stroke (Eleftheriou et al., 2012). It may be in these patients another mechanism is causing increased incidence of thromboembolic events. In addition, an inverse linear relationship between TF and TFPI activity seen in healthy controls was completely absent in paediatric IBD patients, suggesting an important part of regulating thrombosis has been lost. These findings are surprising, and need to be confirmed in a larger cohort compared with age and sex-matched controls to confirm these results are not artefacts of an unconsidered confounding variable.

Chapter 8 addressed the question of evidence of structural arterial disease in paediatric IBD by looking at carotid-femoral PWV. Once age, a well-known positive predictor of PWV (Collaboration, 2010) had been accounted for in multivariate regression analyses, only HDL levels remained a significant predictor. Presence of IBD had no significant effect on PWV in these patients, which suggests if there is any change in vascular stiffness in paediatric IBD, it is too small to be detected easily by PWV. The fact that carotid-femoral PWV is increased in adult IBD (Zanoli et al., 2016) means that while these changes are too small to be detected early on in disease, as IBD progresses they become detectable. Following paediatric IBD patients longitudinally as they age could give insights into when the vasculature starts to stiffen prematurely, and whether these changes occur gradually, or step increases in PWV are seen during significant disease flares.

Chapter 9 investigated the levels of many circulating cytokines in paediatric IBD, and while a variety of pro-inflammatory cytokines were found to be raised, all but IL-12p40 were only

found to be raised by 1-10pg/mL. While statistically significant, these differences are small and it is debateable as to the physiological relevance of increases in this range. Rather than being a biomarker for disease, these cytokines should instead be considered potential therapeutic targets, as discussed in the chapter.

An interesting feature of the results is highlighted in the differing conclusions drawn for IL-17A, sICAM-1 and sVCAM-1, depending on the use of multivariate linear regression. When looking at changes in median values between controls and paediatric IBD patients, all demonstrated an increase in IBD. This fits with previous work in IBD in the case of IL-17A (Cătană et al., 2015), and fits the hypothesis of vascular injury in the case of sICAM-1 and sVCAM-1, both of which are also associated with developing type-2 diabetes (Julia et al., 2014). However, when taking into account confounding variables in multivariate linear regression, there was no longer an association with IBD with these cytokines and a negative association with age. Considering the adolescent controls are on average older than the IBD patients means that we cannot be sure if age is actually a negative predictor of these cytokines, or whether IBD is in fact a positive predictor and results have been skewed by this difference in age.

10.2 Conclusions

Overall, the clinical study into possible causes of increased lifetime thromboembolic complications in paediatric IBD was a success, with evidence shown of endothelial injury, increased circulating MPs, altered thrombin generation and TF/TFPI activity, and an increase in various circulating pro-inflammatory cytokines. Combined with the demonstrated effects *in vitro* of IBD-related cytokines on MP release from endothelial cells, this suggests the increased inflammation associated with IBD is driving MP release and endothelial dysfunction. While changes in vascular stiffness were not seen, the fact they are seen in an adult population shows that these early changes in even well-managed paediatric IBD could be leading to the vascular stiffness changes and increased risk of cardiovascular events seen later in life.

While this study shows interesting changes to the vascular endothelium, circulating MPs and functional changes to thrombin generation and TF activity, the study is underpowered to properly stratify any of these effects by disease subtype, treatment regime, or duration of disease. In addition, the inclusion of only mildly active IBD patients means that the effects of more severely active disease can't be investigated. Careful consideration as to the drawbacks of the controls recruited had to be made as well, with their higher age than the recruited IBD patients potentially affecting many inflammatory parameters investigated. However, the difficulty of recruiting paediatric controls meant this was a compromise worth taking. The addition of multivariate linear regression modelling to take into account confounding variables also helped to ameliorate this drawback, by allowing potential confounding variables to be accounted for.

10.3 Future work

10.3.1 Clinical Studies

The results seen in this small observational comparative study certainly warrant further investigation, which could be done one of two ways. Either a much larger comparative study could be performed with properly age and sex matched controls, to confirm the findings already seen and then to further stratify responses, or a longitudinal study could be performed. This would allow tracking of changes in all investigated parameters, allowing differences to be seen in active disease and remission, and potentially the progression of vascular stiffness with age. A longitudinal study also allows the possibility of correlating these markers with disease flares, to investigate if any of these markers could help predict the next period of active disease. If this is the case, treatment could be escalated before a flare occurs to try and minimise its effects. The fact that vascular, endothelial and MP changes have all been seen in patients in remission shows the sensitivity of these markers, and any that aid in prediction of a flare would be very useful in a clinical context.

10.3.2 mRNA/miRNA/proteins in MPs

While MPs have been phenotyped by antibody surface staining in flow cytometry and changes in subsets have been shown, there is far more potential for identifying and classifying MPs than by their surface markers. As techniques to detect proteins and nucleic acids have become more sensitive and precise, it has become possible to characterise them within MPs. It has been shown in an *in vitro* endothelial model of MP release that incubation with TNF-α drastically alters the amount of a variety of miRNAs compared to control MPs (Alexy et al., 2014). This could be a mechanism by which cells change gene expression in a distal or systemic fashion. Analysis of the miRNA content of MPs in diabetes has highlighted how they could have a significant role in vascular physiology and disease (Alexandru et al., 2016), but they have not yet been analysed in IBD. Showing changes to known miRNA that affect the vascular endothelium would be further evidence and a novel mechanism for the endothelial dysfunction demonstrated by increased CECs.

In addition to nucleic acid content, MPs have also been shown to contain a variety of proteins derived from their parent cell (Milioli et al., 2015). Proteomic analysis of MPs is comparatively in its infancy, but the potential for huge datasets and detailed information on the contents of MPs means they are a prime candidate for biomarker identification (Harel et al., 2015) . The combination of proteomic and genomic strategies in MP analysis has huge potential to expand our knowledge of IBD pathogenesis, however the huge datasets involved would require careful bioinformatics to fully utilise any data.

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