MICROPARTICLES AND THEIR ROLE IN THROMBOSIS IN BEHÇETS SYNDROME AND THEIR POTENTIAL AS A BIOMARKER FOR THROMBOTIC RISK

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DECLARATION

I hereby declare that, as the author of this thesis, all of the work reported was performed by myself. Assistance provided by other persons has been acknowledged and all previous work has been identified and acknowledged. This work is original and has not been submitted for any other degree.

Emon Khan  **Date 2nd June 2017**

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Abstract

Thrombosis is common in Behçet's Syndrome (BS), and there is a clear need both for a better understanding of causation and for biomarkers to enable thrombotic risk assessment. I set out to determine whether plasma microparticles (MP), particularly tissue factor expressing (TF+ MP) were increased in BS. Additionally, I investigated whether MP expressing tissue factor pathway inhibitor (TFPI) were also evident. Finally, I investigated whether these MP had a functional consequence role.

MP were prepared from 88 BS patients and 72 healthy controls. The BS group contained 21 patients with a history of thrombosis (Th+) and 67 without (Th-). MP were identified by size and annexin V binding using flow cytometry, and were further analysed with antibodies to surface antigens. Assays used to assess the function of MP were an in-house coagulation lysis assay, calibrated automated thrombin generation assay and Factor Xa generation assay.

Total MP numbers were increased in BS compared to HC, as were MP expressing TF and TFPI (all $p < 0.001$). Amongst BS patients, the Th+ group had increased total and TF positive MP (both $p < 0.001$) compared to the Th- group, but a lower proportion of TFPI positive MPs ($p < 0.05$). Consequently, the ratio of TFPI to TF MP counts (TFPI/TF) was significantly lower in Th+ *versus* Th- BS patients ($p < 0.001$), with no history of clinical thrombosis in BS patients with a TFPI/TF MP ratio > 0.7. Functional assays revealed no correlation with MP count.

I conclude that MP expressing TF are increased in BS, more so in patients with thrombosis. An imbalance between microparticulate TF and TFPI may be pathophysiologically important for thrombosis in BS and may allow improved identification and appropriate treatment of thrombotic risk.

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Abbreviations

AnV	Annexin V
APC	Activated Protein C
APS	Antiphosholipid Syndrome
AT	Antithrombin
AUC	Area Under the Curve
BS	Behçets Syndrome
CAT	Calibrated Automated Thrombin generation
CRF	Clinical Research Facility
CRP	C-reactive Protein
DMARDs	Disease-Modifying Anti-Rheumatic Drugs
EN	Erythema Nodosum
ERAP	Endoplasmic Reticulum Aminopeptidase
ESR	Erythrocyte Sedimentation Rate
ETP	Endogenous Thrombin Potential
EULAR	European League Against Rheumatism
FACS	Fluorescence-Activated Cell Sorting
FFP	Fresh Frozen Plasma
GI	Gastrointestinal
GWAS	Genome Wide Association Studies
HC	Healthy Controls
HLA	Human Leucocyte Antigen
HUVEC	Human Umbilical Vein Endothelial Cells
IFN	Interferon
IL	Interleukin
ISG	International Study Group

IVC	Inferior vena cava
LMWH	Low Molecular Weight Heparin
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MICA	MHC Class I chain related
MP	Microparticles
MPPP	Microparticle Poor Plasma
OR	Odds Ratio
PAI-1	Plasminogen Activator Inhibitor-1
PAR	Poly(ADP) Ribose
PDI	Protein Disulphide Isomerase
PL	Phospholipid
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PSGL1	P-selectin Glycoprotein Ligand-1
RCT	Randomised Controlled Trial
ROTEM	Rotational Thromboblastometry
SLE	Sytemic Lupus Erythematous
SNP	Single Nucleotide Polymorphism
STAT	Single Transducers and Activators of Transcription
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
Th	Thrombosis
TNF	Tumour Necrosis Factor
tPA	Tissue Plasminogen Activator
TPMT	Thiopurine-S-Methyltransferase

UBAC	UBA domain Containing
V_{MAX}	Maximum Rate of Thrombus Generation
VTE	Venous Thromboembolism

1 Introduction

1.1 Haemostasis and thrombosis

Haemostasis occurs at the site of vessel injury and is a combination of vasoconstriction, platelet aggregation and coagulation, that allows the body to maintain the integrity of the circulatory system. When the vessel wall is injured, vasoconstriction occurs, reducing blood flow to the injured area and prevents excessive blood loss. There is also exposure of several vessel wall components such as collagen, fibronectin, von Willebrand Factor and tissue factor. This becomes the site of platelet aggregation, and this process is dependent on these vessel wall factors and several platelet-derived factors, such as agonists, receptors and adhesive proteins [1]. This aggregation of platelets allows a plug to form, but coagulation is required to stabilise this plug. Coagulation is the process by which the blood changes from a liquid to a gel, as a result of fibrin formation. Fibrin is deposited around and within platelet aggregates to stabilise the plug and maintain haemostasis. These processes occur simultaneously, preventing haemorrhage, which can lead to excessive blood loss, causing considerable morbidity and mortality. Another factor that contributes to the process of haemostasis is blood flow and shear [2, 3]. This leads to differential expression of adhesion molecules on the surface of the endothelium. Haemostasis is controlled by several regulatory mechanisms in the blood and vessel wall, preventing this process from occurring without injury [4].

Thrombosis is the pathological manifestation of the above process, when dysregulation of the haemostatic system occurs due to an abnormality in the constituents of the blood, vessel wall, blood flow or a combination of these. This constitutes Virchow's triad, and is the basis of the study of thrombosis, both the manifestations and causes. Thrombosis can occur in the veins or the arteries. When

it occurs in the veins it is commonly called venous thromboembolism (VTE). It can cause considerable morbidity and death, and has an incidence of approximately 1 in every 1000 of the general adult population per annum [5, 6]. 50-60% of VTE are due to known risk factors, such as malignancy, thrombophilia, immobility or surgery [7]. In arteries, thrombus often occurs because of atherosclerotic plaque rupture, and is also dependent on blood flow [8].

Coagulation is an essential part of thrombosis. How it occurs and the control of this process, has been the subject of study over many centuries. The Ancient Greeks were the first to investigate coagulation, and Plato coined the term ‘fibrin’ in relation to coagulation [9]. The Ancient Greeks and Romans first attempted to control haemorrhage by using ligatures in the field of battle [10]. In the middles ages, following the lead of Arab surgeons, it became common practise to use boiling oil to control haemorrhage. It was only when military surgeon Ambroise Paré (1510 – 90) ran out of boiling oil during a battle that this method was abandoned. He started using ligature instead, and found that there were better outcomes with this method [10].

Coagulation was thought to be the function of red cells, but it remained a mystery as to how it occurred. William Hewson (1739 – 1774) first demonstrated in 1771 that clotting was a function of ‘coagulable lymph’, as he called plasma, by the addition of neutral salts removing plasma thereby delaying clotting [11]. He was also able to precipitate a white substance from plasma when heating it to 50˚C, which he reported was responsible for clot formation, later identified as fibrin [10].

There was no significant progression for the next 50 years or so until Buchanan in 1836 showed that the addition of white blood cells and serum to red blood cells led to clot formation, thereby extending the findings of Hewson [12]. This meant that there was another factor that led to the conversion of fibrinogen to fibrin. Schmidt’s

discovery of fibrinogen in the mid- to late-19th century was the initial step leading to the discovery of thrombin. He discovered a water-soluble residue that, when added to a solution containing fibrinogen, led to rapid clot formation. This water-soluble residue was thrombin [13]. He also postulated the existence of prothrombin, since circulating thrombin would cause the blood to clot [9]. It was from here that coagulation theory was first developed.

1.1.1 Coagulation cascade

1.1.1.1 Background

Coagulation occurs via coordinated proteolysis of a series of zymogens. This occurs in a rapid manner, to prevent significant haemorrhage, and is proportionate to prevent obstruction to blood flow. These zymogens, or clotting factors, have been discovered over time following initial clotting theory of Morawitz in 1905 [14].

Coagulation was first described as a waterfall cascade of zymogen activation by Davie, Ratnoff and MacFarlane in 1964 [15, 16]. They showed how the fibrin clot formed by commencing at FXII. By this stage, the terms 'intrinsic' and extrinsic' had come into use. The intrinsic cascade commences when there is contact activation with a negatively charged surface, with the main factors being XII, XI, IX and VIII. The extrinsic system is triggered by exposure of tissue factor (TF) to blood, and its interaction with FVII. TF, also known as coagulation factor III or tissue thromboplastin, is a 47kDa glycoprotein that was first purified from human brain tissue in 1985 [17]. It is the most potent activator of the coagulation cascade. FX, FV, prothrombin, fibrinogen and FXIII make up the common pathway. Once factors are activated, they are annotated as such, for example activated FX becomes FXa.

The traditional view of the coagulation cascade being split into intrinsic and extrinsic pathways, and ending in the common pathway remains widely used. Tests of the intrinsic and extrinsic coagulation pathways, the prothrombin time (PT) and activated partial thromboplastin time (APTT) respectively, remain in common usage decades after their invention [18]. Although this is a useful way of thinking about the coagulation cascade, it does not represent what occurs *in vivo*. For example, low levels of FXII, high molecular weight kininogens (HMWK) and prekallikrein lead to large elevations of the APTT, without any significant haemorrhage in humans. FVIII and FIX deficiency, however, which lead to haemophilia, manifest a similar magnitude in APTT rise, but with significant haemorrhage. This occurs despite the extrinsic pathway being intact. Similarly, those with FVII deficiency have a severe bleeding tendency, despite the intrinsic pathway being intact. This demonstrates that both pathways are important for effective haemostasis.

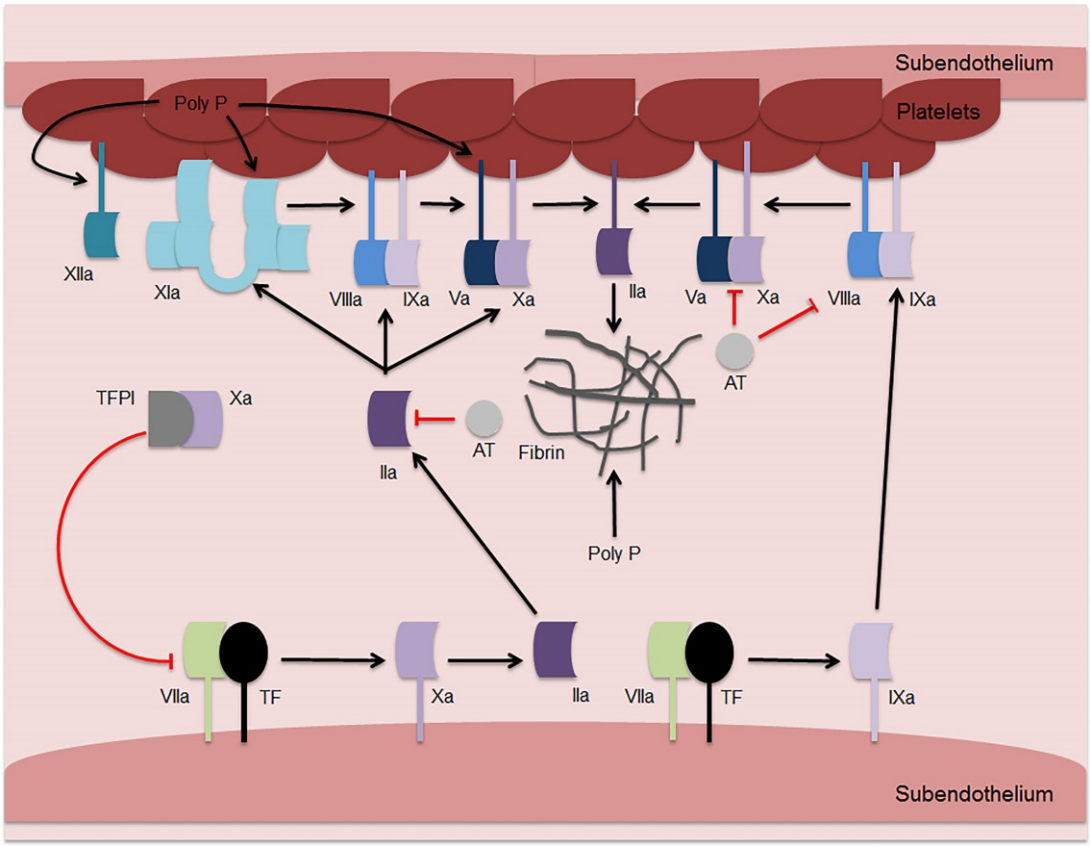
Davie and Ratnoff first commented that the ‘intrinsic system probably overlaps with the extrinsic system at the level of Stuart Factor (FX)’ [15]. This is the start of the common pathway that leads to thrombin generation. FX was first confirmed in investigation of two patients with presumed FVII deficiency. The first was a 22-year-old woman with a history of bleeding following dental extractions and tonsillectomy, with prolonged prothrombin time, whose bleeding tendency was corrected by the addition of plasma from patients on vitamin K antagonists [19]. The second paper gave the name Stuart Factor to FX. This was a case history of a 36-year-old who was initially thought to have FVII deficiency, but when their plasma was mixed with the plasma of a patient with proven FVII deficiency, there was correction of the PT for each patient [20].

FXa’s main function is as the first step in the common pathway, leading to the generation of thrombin from prothrombin. FXa is also known to activate factors V

[21, 22], VII [23, 24] and VIII [25, 26]. The impact of FXa on the activation of these factors is variable. This pathway of FV activation is not as significant as that of thrombin [27], whilst low concentrations of FXa are important in the activation of FVII by TF [28] and FXa and thrombin are both important in the activation of FVIII [29]. These interactions provide a positive feedback loop for FXa generation and a further link between the intrinsic and extrinsic systems, thereby a link between the initiation phase and, the amplification and propagation phases of coagulation.

Haemostasis is now seen as a cell-based process that consists of three phases: initiation, amplification and propagation [30]. The initiation phase is dependent upon the presence of TF and is due to activation of the extrinsic system. The small amount of thrombin and FIXa generated in the first phase leads to the amplification phase, which then leads to the formation of tenase and prothrombinase complexes, and the propagation phase. This model demonstrates the controls in place that allow coagulation to occur in a proportionate way to maintain the integrity of the vascular system and protect vital organs. Below I will summarise the steps in turn and expand on specific aspects of each step, including the factors that play a vital role in each. This is shown below in Figure 1-1.

Figure 1-1 The coagulation cascade



There are three phases to coagulation: initiation, amplification and propagation. The initiation phase commences with endothelial damage, leading to exposure of tissue factor (TF) to the blood where it activates FVII. The TF-FVIIa complex then activates FX, which in turn activates FII (prothrombin). Small amounts of thrombin (FIIa) activates FXI, FVIII and FV, in the amplification phase. This then leads to the propagation phase, which requires the FIXa activated by the TF-FVIIa complex in the initiation phase. FIXa translocates to the activated platelet surface to form the tenase complex with FVIII. This then converts FX to FXa, which binds to FVa to form the prothrombinase complex, leading to thrombin generation on the surface of activated platelets and fibrin formation. The propagation phase is also facilitated by polyphosphates (PolyP), which only directly activate the contact system, by activating FV, FXI and FXII, and inhibits clot lysis. There are fibrinolytic regulators of the Coagulation cascade. Tissue factor pathway inhibitor (TFPI), inhibits the translocation of FXa from TF-bearing cells onto activated platelets, and forms a complex with FXa to inhibit the function of the TF-FVIIa complex. Antithrombin (AT) inhibits the function of thrombin, FIXa and FXa (From Versteeg, 2013)[4].

1.1.1.2 Initiation phase

This is a TF-dependent process that occurs on the surface of TF-bearing cells, many of which are present outside the vasculature. (TF is discussed in greater detail in section 1.1.2.2.1). TF forms a complex with FVIIa, and the main substrates

of the TF-FVIIa complex are FX and FIX. Normally, FVII is kept relatively inactive in a zymogen like conformation, and it is unable to significantly convert FX to FXa without the presence of TF [31]. FXa can interact with FVa on TF-bearing cell surfaces that contain significant amounts of phospholipid to form prothrombinase complexes, which leads to platelet activation [32]. Prothrombinase development on the surface of the phospholipid containing TF-bearing cells have been shown to play a significant role in coagulation. This has been shown in a study of how tick saliva prevents coagulation during a blood meal. There is an antigen in tick saliva that specifically inhibits FXa activation of FV at the B domain, showing that FV activation via FXa plays a role in the intiation phase of coagulation [33].

TF-FVIIa is known to activate FIX [34]. FIXa can translocate from TF-bearing cells to the platelet surface, where amplification and propagation phases occur. This transit through the fluid phase is relatively uninhibited, however, FXa must remain on the surface of TF-bearing cells to remain active. If FXa enters the fluid phase then it is rapidly inactivated by tissue factor pathway inhibitor (TFPI) and antithrombin [35]. FXa forms a complex with TFPI, which can bind with TF-FVIIa complex, thereby inhibiting further conversion of FX to FXa on the TF-bearing cells providing an extra control on thrombin generation.

1.1.1.2.1 Tissue factor

Prior to its formal identification, the existence and importance of TF was postulated in the 19th century. In 1834, de Blainville showed that intravenous injection of brain tissue into an animal, caused almost immediate vascular occlusion and death [9]. It is also central to Morawitz's original theory of coagulation in 1905 [10, 14].

TF is found in areas such as the cerebral cortex, renal glomeruli, cardiac myocytes and the adventitia. TF remains inactive until there is vessel compromise, exposing it to the circulation where it is ready to initiate coagulation, like a 'haemostatic envelope' [36, 37]. These are 'high TF' areas and are protected from catastrophic bleeding. TF's significant role in haemostasis remained elusive for several decades in the 20th century, because there were no models or clinical cases of TF deficiency. It is vital for embryogenesis, with TF gene knockout mice all shown to die *in utero* [38-40]. Also, via protease activated receptor (PAR) 2 signalling, it leads to placental development [41].

TF triggers the extrinsic pathway of the coagulation cascade. It is the most potent activator of the coagulation cascade, increasing the catalytic ability of FVIIa to convert FX to FXa 2-million-fold [42, 43]. The domain structure of TF includes an N-terminal extracellular region (residues 1-219, TF_{1-219}), a 23 amino acid transmembrane sequence (residues 220-242), and a cytoplasmic tail containing palmitate and stearate bound to cysteine residues 243-263 [44, 45]. FX is cleaved by the extracellular region of TF in the presence of phospholipid, which is diminished by prothrombin fragment 1 and is abrogated by the removal of phospholipids [46]. FXa generation increases with phospholipid concentration even with the same concentration of TF_{1-219} and FX, showing that the presence of anionic surfaces for coagulation factors to bind to is important to generate thrombin [46]. Activation of FX is achieved by initial recognition of FX by TF-FVIIa complex. FX recognition by the TF-FVIIa complex is dependent on cofactor residues lysine 165 and 166 that make up sidechains present in the carboxyl half of the extracellular domain of the complex [47]. Therefore, a discrete defect that leads to the deletion of these sidechains, thereby diminishing the rate of proteolytic activation of FX by TF-FVIIa complex. This defect leads to a significant decline in the activation of FX,

which occurs with and without the presence of phospholipids, confirming that these residues are important in the recognition of FX by TF-FVIIa complex [47].

The efficient hydrolysis of FX is achieved by residues 157 – 167 of the TF-FVIIa complex, which also promote the conversion of FVII to FVIIa [48]. FX also appears to interact with the TF-FVIIa complex at a site on the C-terminal fibronectin type III domain of TF, along with FIX [49]. This study showed that a particular residue Lys159, was part of the main interaction region for FX and IX, which was not found in a previous study by Ruf *et al* [48, 49]. It has also been shown that the TF region interacting with FXa to activate TF, also interacts with FX to activate FVII, thereby serving a dual role [28]. It is the Gla and first epidermal growth factor-like domains of FX that interacts with the TF-FVIIa complex, and this area also interacts with protein C impairing the activation of FX by TF-FVIIa complex [50, 51].

TF provides a two-way link between thrombosis and inflammation. Inflammatory activation of monocytes/macrophages by endotoxin or inflammatory cytokines induces TF expression [52, 53], while thrombin generation via TF expression leads to the inflammatory activation of cells via PARs [54, 55]. It is through this combination of actions that TF is considered to play a vital role in diverse pathologic processes including atherosclerosis, thrombosis, sepsis and tumour growth [56-59].

1.1.1.2.2 Tissue factor pathway inhibitor

When the classical theory of coagulation was developed in the early twentieth century, the focus was on the process of coagulation, rather than any controlling mechanism. In 1922, Loeb wrote that [60, 61]:

> The effect of a mixture of serum and tissue extract on fluoride plasma, and the curve representing the coagulation time of the plasma as a function of the time during which the

> serum and extract were allowed to stand at room temperature before being added to the plasma, can be explained if we assume that two processes go hand in hand in the mixture; namely, the formation of a substrate accelerating the coagulation of the plasma and of a substrate inhibiting in some way the action of the coagulating substance. (Quoted from Loeb, 1922)

There were numerous attempts to separate out this new 'antithromboplastin' factor. Tocantins did a significant amount of work in the 1940s and 50s, and was able to extract 'a heat-labile lipid inhibitor of coagulation from brain tissue', which although not as potent as heparin had a 'more long-lasting effect' when injected [62].

Further work showed that this new anticoagulant acted in a calcium dependent manner in inhibiting the action of TF in serum and plasma [63]. Studies confirmed that there was an 'antithromboplastin' in the serum inhibiting the effect of TF, supporting the theories of Tocantins in the late 1940s, that [64, 65]:

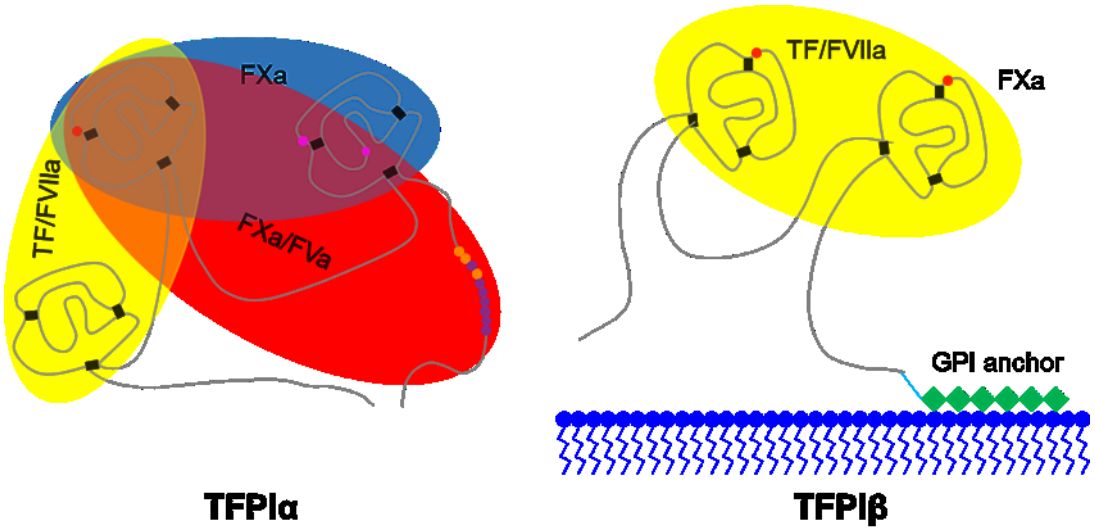
> Fluidity and coagulability are properties of the blood that are in a sense antagonist to each other. To understand what promotes coagulation, one must be familiar with the factors that discourage this change and keep the blood in a fluid state. (Quoted from Tocantins, 1946)

Subsequent studies showed much of this inhibitory activity was associated with lipoproteins, confirming that the inhibition of TF function was calcium-dependent and could be abrogated by the removal of calcium, but also required the presence of FVIIa and FXa to inactivate TF activity [66-68]. It was the work of Broze and Miletich that first isolated TFPI, or tissue factor inhibitor as they called it, as a M_r 38,000 band by polyacrylamide gel electrophoresis from a human hepatoma cell line, following a four-step process including chromatography [69]. A series of clotting studies proved the inhibitory action and that it was at its greatest in the presence of FVIIa and FXa. It was also soon shown that this lipoprotein associated inhibitor,

acted quickly after the generation of FXa and FIXa in the TF-dependent system to inhibit TF activity in a calcium dependent manner [70].

TFPI is a single chain Kunitz-type proteinase inhibitor and is the primary inhibitor of the initiation phase of the coagulation cascade, which consists of the TF-dependent pathway [71, 72]. It is a large glycoprotein that initially inhibits FXa directly, and then inhibits the TF-FVIIa complex in FXa-dependent manner [72, 73]. There are two isoforms of TFPI that are generated from alternative mRNA splicing (Figure 1-2) [72]. TFPI-α, which has three tandem Kunitz-type domains, interacts with TF-FVIIa, FXa and prothrombinase complex [74]. Kunitz-2 interacts with FXa, whilst Kunitz-1 inhibits with TF/FVIIa complex in FXa-dependent manner [75-77]. For inhibition to occur, the sequential binding of TFPI to FXa and then to TF/FVIIa to form a quaternary complex is vital, with the initial binding to FXa being the rate limiting step [78]. This initial step is enhanced by protein S, which acts in the protein C pathway, by being a co-factor in activated protein C (APC) mediated inactivation of FVa and FVIIIa [79]. Protein S also acts to enhance the interaction between TFPI and FXa in an APC-independent manner [80, 81]. It has been shown that protein S interacts with the Kunitz-3 domain of TFPI-α and inhibits FXa [82]. Only TFPI-α inhibits the prothrombinase complex by interaction of the K2 domain with FXa and the C-terminal and the acidic region of the B domain of FV [74, 83]. The α isoform can circulate free, but the combination of C-terminal and Kunitz-3 domains allow for cell surface binding, especially to lipoproteins [84].

Figure 1-2 TFPI isoforms and structure



This figure shows the structures of the α and β isoforms of TFPI. The grey lines represent the amino acid chains. The α isoform can circulate free or is bound to lipoprotein (LDL and HDL) and contains three tandem Kunitz-type domains (K1 – 3) with a C-terminal region. The β isoform contains two Kunitz-type domains with the K3 and C-terminal regions of the α isoform replaced by an unrelated C-terminal region that directs the attachment of the glucosylphosphatidylinositol anchor to the membrane. The small red circles in the K1 and K2 domains bind to FVIIa and FXa. The small pink circles bind protein S. The small orange and purple circles are basic and hydrophobic residues that bind the acidic region of FVa. The shaded yellow area is for inhibition of TF/FVIIa, the blue area FXa and red area FXa/FVa. (From Wood, 2014) [72].

The β isoform differs from the α isoform in that the Kunitz-3 and C-terminal domains are replaced by a different C-terminal region that attaches to the cell membrane via a glycophosphatidylinositol anchor [85]. This isoform makes up most of the TFPI available, so inhibiting FXa and TF-FVIIa on the endothelium [76].

Endothelial cells and megakaryocytes primarily produce TFPI, although it has also been shown to be synthesised by other cells such as monocytes [86-88].

Deficiency in TFPI leads to a pro-thrombotic state, as there is decreased inhibition of the TF-dependent pathway of the coagulation cascade [73]. This has been confirmed in studies of the general population, where low overall TFPI levels lead to an increased incidence of thrombosis [89]. Additionally, it has been shown in vitro that blockade of TFPI leads to increased sensitivity in thrombin generation assays

[90]. Heparin is known to stimulate the release of TFPI *in vitro* from an endothelial cell line, as well as increasing mRNA, in a dose- and time-dependent manner [91].

It appears that total TFPI deficiency is incompatible with life. In mice, homozygous for TFPI Kunitz-1 domain deletion, there was accelerated yolk sac haemorrhage leading to death of mouse embryos [92]. There are no known cases of total TFPI deficiency, suggesting that it causes lethality in humans as well. In those with low TFPI levels, especially below the 10th centile, there is an increased risk of cardiovascular disease and venous thrombosis [93].

TFPI plays an important role in the coagulation system, by preventing unfettered thrombin generation, which can lead to thrombosis. It has been shown to be vital to life, since total TFPI deficiency leads to death in animal models.

1.1.1.3 Amplification phase

The end-point of the initiation phase is the generation of thrombin. This is a multi-functional serine protease that has numerous haemostatic and non-haemostatic roles. The thrombin generated in the initiation phase is important, since it leads to activation of other factors, which allows the coagulation cascade to proceed on the surface of activated platelets predominantly down the intrinsic pathway. In *in vitro* studies consisting of TF-bearing monocytes, platelets and inactivated VIIa and Xa factors, there is no platelet activation, thereby confirming the pivotal role of the small amount of thrombin generated in the initiation phase in subsequent phases of coagulation [94]. Thrombin also activates platelets leading to the release of adenosine diphosphate (ADP) and thromboxane A_2 [95].

The importance of thrombin in clot formation was established in the 19th century and was defined in Morawitz's initial theory of clotting [12, 14]. Thrombin not only leads

to the conversion of fibrinogen to fibrin, but also activates factors V, VIII and XI [96-98]. This is preparation for the next phase of coagulation.

1.1.1.3.1 Haemophilia

The model above is important in explaining the importance of both arms of the coagulation cascade. The extrinsic pathway is the trigger for coagulation, and takes place on TF-bearing cells. The intrinsic pathway takes place on activated platelets and appears to be essential for clot stability [99]. This is shown in haemophilia, and explains why there is a bleeding tendency in haemophiliacs.

Since FXa is unable to safely transit the fluid phase without inactivation from TFPI and antithrombin, the FXa production on the surface of activated platelets is required for thrombin generation. This cannot occur without the assembly of the tenase complex, and this does not occur in haemophilia.

FIXa can activate FXa, but FVIIIa increases the rate of FXa generation 200,000-fold in combination with FIXa [100]. The defect in haemophilia is not just of FXa generation on activated platelets, but of unfettered fibrinolysis. Thrombin activatable fibrinolysis inhibitor (TAFI) is important in regulating fibrinolysis, particularly in low TF areas of the body. The impact of low FVIII and FIX levels are much more pronounced in these areas, such as the skeletal muscle and joints, because there is not enough thrombin generated for clot formation, and for TAFI production. Fibrinolysis inhibition has been shown to be downgraded because of reduced TAFI levels in low TF areas, and is further reason for impaired clot stability in haemophiliacs [101]. Also, morphological study shows that the central aspect of the clot has little fibrin deposition at 2 hours in patients with haemophilia A [102]. The

combination of unfettered fibrinolysis and the failure of the tenase complex, leads to unstable clots.

1.1.1.4 Propagation phase

The propagation phase occurs on activated platelets. FVIIIa is a co-factor to FIXa and forms the tenase complex, and it mediates the binding of FIX and FX to the surface of platelets [103], thereby promoting the activation of FX and generating thrombin [104]. The FIXa comes from two sources: the first is from the surface of TF-bearing cells in the initiation phase and the second is on the surface of activated platelets by FXIa.

Since FXa is unable to transit the fluid phase without being inactivated by TFPI or antithrombin almost all the FXa in the propagation phase must be generated on the surface of activated platelets [104]**.** FVa then forms the prothrombinase complex with FXa, Ca^{2+} and phospholipids, significantly increasing the activity of FXa to convert prothrombin to thrombin [96]. There is still circulating TFPI, but at physiologic concentrations, it is unable to inhibit prothrombinase activity on the surface of activated platelets [105].

FXa's main function is as the first step in the common pathway, leading to the generation of thrombin from prothrombin. FXa is also known to activate FV [21, 22], FVII [23, 24] and FVIII [25, 26]. The impact of FXa on the activation of these factors is variable. The activation of FV by FXa is not as significant as thrombin's activation of FV [27], whilst low concentrations of FXa are important in the activation of FVII by TF [28] and FXa and thrombin are both important in the activation of FVIII [29]. These interactions provide a positive feedback loop for FXa generation and a further

link between the intrinsic and extrinsic systems, thereby a link between the initiation phase and, the amplification and propagation phases of coagulation.

1.1.1.4.1 Thrombin

Unlike deficiencies in FXII, kallikrein and HMWK, deficiency in FXI leads to a bleeding tendency and is inherited in a predominantly autosomal dominant fashion most often in Jewish people [106]. Most often it is discovered in those with bleeding after surgery. These patients also show that FXI is activated in a manner independent of FXII, kallikrein and HMWK. FXII has a negligible contribution to normal haemostasis and FXI activation *in vivo* [99]. Thrombin has been shown to activate FXI, independent of FXII, with the assistance of glyocosaminoglycans or proteoglycans [107, 108]. FXIa activates FV and FVIII [109], IX [110], X [111] and enhances TF-FVIIa activity by inactivating both TFPIα and β, on platelets and endothelial cells respectively [112]. FXI has become a target for therapy, with FXI shown to increase the risk of postoperative venous thrombosis, and FXI antisense oligonucleotide reducing the risk of venous thrombosis following elective unilateral knee arthroplasty [113]. This suggests a significant role for FXIa in thrombosis.

The other actions of thrombin in the propagation phase are involved in clot stabilisation. Thrombin activates FXIII, and FXIIIa leads to 'gelation' of fibrinogen and catalyses the formation of protein-to-protein side bridges, thereby stabilising the fibrin clot [114, 115]. It also activates TAFI, which is important in impairing fibrinolysis further promoting clot generation.

Thrombin's actions on platelets are primarily mediated through protease activated receptors (PARs). PARs are G-protein coupled, seven-trans-membrane receptors that are cleaved by proteases, which exposes a tethered ligand leading to activation

[54, 116]. Thrombin primarily mediates its platelet procoagulant activity via PAR1, but also interacts with PAR4 [117, 118]. PAR4 is a low affinity receptor for thrombin and tends to require higher levels of thrombin for activation, which seems to occur later in the propagation phase of coagulation [119].

ADP-induced platelet aggregation requires coactivation of the P2Y1 and P2Y12 receptors, and it is necessary for fibrinogen to be bound to $\alpha_{IIb}\beta_3$ receptor, which induces platelet aggregation, for this to occur. ADP-induced platelet aggregation and thromboxane A_2 release is inhibited in the presence of SC49992, a fibrinogen receptor antagonist, in a concentration dependent manner [120]. This shows that ADP is dependent on platelet aggregation before causing conformational change in platelets.

1.1.1.4.2 Polyphosphates

Over recent years, it has become apparent that the action of thrombin and other coagulation factors in the propagation phase is assisted by polyphosphates. These are inorganic, linear polymers of many orthophosphate units linked by phosphoanhydride bonds that are found in every cell, including those of bacteria, plant and animal [121]. In platelets, they are also stored within the dense granules and are released, alongside serotonin, following thrombin stimulation [122]. Polyphosphates provide an anionic surface that allows the binding of coagulation factors, which promotes the propagation phase of coagulation on the surface of activated platelets. They primarily act in the intrinsic coagulation pathway.

Polyphosphates are of variable lengths and there are differing roles for longer and shorter chain versions. Longer chain polyphosphates can activate the FXII driven coagulation system and promote platelet-driven fibrin formation, which is

independent of FXI [109, 123]. They also have an additional role in abrogating fibrinolysis, with chains longer than 25 orthophosphates impeding TFPI action and longer chain polyphosphates also inhibiting fibrinolysis in a TAFI-dependent manner [124]. In both processes, increasing concentration of polyphosphates also promoted activity and when TAFI-deficient plasma was used in a lysis assay, fibrinolysis was impaired. It appears that thrombin and polyphosphates, via its activation of FXII, play a role in TAFI activation and the delay of fibrinolysis. FXIa is known to activate FV, but polyphosphates have also been shown to activate FV via thrombin and FXa [111, 124].

Shorter chain polyphosphates can accelerate the activation of FV by FXIa, by binding with high affinity to FV and enhancing FV cleavage by FXIa [125]. This is important, since the small amount of thrombin generated in the initiation phase is not adequate for FV activation and formation of prothrombinase complex, so this is another pathway to thrombin generation in the amplification and propagation phases on activated platelets.

Polyphosphates also promote feedback activation of FXI by thrombin, so inhibition of polyphosphates and FXIa during clotting of human plasma on TF-bearing and collagen surfaces in a flow system, have been shown to reduce the rate of thrombosis [126]. This interaction between polyphosphates, thrombin, FXI and FXII, promotes the intrinsic coagulation cascade. This interaction also promotes clot stability, which is the most significant role of the intrinsic system in coagulation. This has been shown in FXII-deficient mice who have had thrombus induced. Intravital microscopy of these mice showed a defect in the formation and stabilisation of platelet-rich occlusive thrombi, demonstrating a role for FXII in clot stability [99].

1.1.1.5 Summary

In summary, the initiation phase is the starter motor for coagulation, by generating a small amount of thrombin that activates other coagulation factors to perpetuate thrombin generation on the surface of platelets. The FIXa generated on the surface of TF-bearing cells is very important, because it can transit the fluid phase and form the tenase complex with FVIIIa, allowing generation of FXa on the surface of platelet, which then forms the prothrombinase complex with FVa. The combination of tenase and prothrombinase complex activity leads to significant thrombin generation, which seems to only occur on activated platelets. The action of thrombin is ably assisted by polyphosphates, which promote clotting factor activation and clot stability. This model also explains why certain disorders of the intrinsic and extrinsic systems can cause significant morbidity.

1.1.2 Thrombophilia

Thrombophilia is an abnormal tendency to develop clots and can be inherited or acquired. There are several studies that suggest an association of thrombophilia with spontaneous (unprovoked) thrombosis, recurrent thrombotic events, thrombosis at an early age, or in those with a strong family history of thrombosis. Inherited thrombophilias include Factor V Leiden gene mutation, prothrombin gene mutation, antithrombin deficiency and deficiencies of protein C and S. Although these have been associated with an increased risk of thromboses, the magnitude of their impact is debatable, such that it is controversial whether routine testing for thrombophilia leads to a reduction in the incidence of first and recurrent thromboses.

Factor V Leiden deficiency is the most common inherited thrombophilia in Caucasian populations and is caused by a specific mutation in FV that results in an

inability to be cleaved by APC [127-129]. This condition, also known as activated protein C resistance, results in increased thrombin generation, leading to a hypercoagulable state. Patients who are heterozygous for this mutation are estimated to have up to a 7-fold risk of thrombosis compared to the general population [128]. This risk increases in those who are homozygotes who have severe APC resistance as a result, by up to 80-fold [128, 130]. It has, however, been shown in a retrospective study that the risk of a first thrombotic episode in those with a family member with thrombosis and Factor V Leiden mutation is low at 0.45 [131].

A study by Poort *et al* showed that the specific mutation G20210A (guanine to adenosine) in the 3'-untranslated region of the prothrombin gene was associated with an increased risk of thrombosis [132]. Confirmation of an association of the prothrombin gene mutation with venous thrombosis was shown in a study of 366 patients recruited between 1994 and 1997 in France [133]. Each patient had an episode of VTE and the odds ratio of prothrombin gene mutation was 4.8 compared to a group of healthy volunteers. There were, however, only 17 patients who had the prothrombin gene mutation in this study. Like Factor V Leiden, this mutation appears to be found predominantly in Caucasians where it is the second most frequent thrombophilia, but is extremely rare in Black and Asian populations [133, 134]. Heterozygote carriers for prothrombin gene mutation are estimated to have a 3 to 4-fold risk of thrombosis [132, 133, 135, 136]. Those who are homozygous for the mutation have similar thrombotic risks to those with homozygous Factor V Leiden deficiency [137].

Protein C and S deficiencies are both relatively rare causes of inherited thrombophilia, occurring in 0.2-0.4% of Caucasian populations, and rarely in other ethnic groups [130, 138, 139]. They have a similar phenotypic presentation, with

most patients' heterozygotes with levels of protein C or protein S <50% [140, 141]. Older studies suggested an increased risk of VTE in those with protein C or S deficiency. Those who are heterozygous for protein C deficiency have a 6 to 10-fold increased risk of thrombosis [142, 143]. Homozygotes are rare, but usually present in infancy with a much more severe phenotype of widespread thrombosis [144, 145]. Like protein C deficiency, homozygotes for protein S deficiency present in early infancy with widespread thrombosis [146, 147]. Protein S heterozygosity is also associated with an increased risk of thrombosis, and more so than Factor V Leiden mutation [148].

Mutations in the antithrombin gene lead to deficiency of its protein and leads to an increased risk of thrombosis. This is found in 0.02-0.04% of the population [136]. Heterozygotes generally have antithrombin levels of 40-60% of normal, and a 5-fold increased risk of thrombosis [149]. Patients with these conditions tend to present from early adulthood onwards with thrombotic events. Homozygosity for antithrombin deficiency has been shown to be lethal in mice and appears to be fatal in utero in most humans, with only a few cases reported in the literature. These almost all presented with severe thrombotic events from childhood [150, 151].

A prospective study of 382 relatives of 84 patients with a history of VTE and protein S, protein C and antithrombin deficiency, also confirmed the increased risk of VTE [152]. Annual incidence of unprovoked VTE was 0.95% in deficient relatives *versus* 0.05% in non-deficient subjects, confirming that these deficiencies confer an elevated risk of thrombosis.

Compound heterozygotes, with more than one inherited thrombophilia have also been reported. Most are heterozygous for Factor V Leiden deficiency, along with either protein C, protein S or antithrombin. These patients appear to have higher risks of thrombosis than those with single mutations, and particularly higher rates of

recurrent thrombotic events [137, 153-155]. A couple of studies have shown that inherited and acquired mutations in the FV and prothrombin genes, and deficiencies in protein C, protein S and antithrombin have all been associated with an increased risk of recurrent thrombosis [133, 153].

Despite this, thrombophilia testing is not part of routine clinical practice in those who are about to commence medications, such as oestrogens, that confer an increased risk of thrombosis or in those who have had a first episode of thrombosis. Guidelines from the British Committee for Standards in Haematology and the British Society for Haematology do not recommend routine thrombophilia testing [156], and testing for inherited thrombophilia has not been shown to reduce the recurrence of venous thromboses [157].

Prospective studies have shown no evidence that checking for thrombophilia following a first episode of thrombosis reduces the risk of recurrent events. A prospective study showed testing for thrombophilia was not useful within two years' of completing anticoagulation, and there was no recurrence in those who had VTE episode following surgery [158]. Kearon *et al* followed up 661 patients with a history of unprovoked VTE and one or more thrombophilia for an average of 2.3 years to assess the risk of recurrent episodes [159]. These patients were given different warfarin regimens, and there was no evidence that patients having one or multiple thrombophilia and an increased risk of recurrent VTE, although there was a trend towards increased recurrence in those with antiphospholipid syndrome [159]. In the study by Mahmoodi *et al* of relatives of patients with deficiencies of protein C, protein S and antithrombin, screening and commencement of thromboprophylaxis may result in reduction of provoked VTE, but the risk of unprovoked VTE did not decline with this approach [152].

Systemic review of 15 studies, showed that recurrence was low in those with a surgery provoked thrombotic event, intermediate for those that was non-surgically provoked and high for those that were unprovoked [160]. This analysis estimated that the rate of recurrence after stopping treatment in patients with symptomatic index VTE provoked by a transient risk factor was 3.3% during the first year and 6.6% during the first 2 years [160]. The highest risk of recurrence was in patients with unprovoked VTE, who had a risk of recurrence that was approximately 2.5-fold that of all patients with VTE provoked by a transient risk factor, 7-fold that of patients with VTE provoked by surgery, and 1.5-fold that of patients with VTE provoked by a nonsurgical trigger [160]. Consequently, it is more relevant to decide whether episodes of thromboses are provoked or unprovoked, to determine the need for thromboprophylaxis, with the possibility of those who have had a provoked episode being overtreated after being found to have inherited thrombophilia.

1.1.3 Inflammation and thrombosis

Although many episodes of thrombosis are the result of known factors, such as immobilisation, post-surgery, thrombophilia or malignancy, a substantial proportion are idiopathic. It is likely that these idiopathic episodes are due to a combination of as yet unidentified acquired and congenital factors, whose cumulative effect causes an alteration in one or more aspects of Virchow's triad of a similar magnitude as that caused by traditional risk factors. Amongst these non-traditional risk factors for thrombosis is inflammation.

The interface of inflammation and thrombosis has been known for over 20 years. C-reactive protein (CRP) has been shown to increase TF expression and procoagulant activity from peripheral blood mononuclear cells [161]. Tumour necrosis factor α has been shown to trigger the intrinsic and common pathway of coagulation following

injection into healthy volunteers [162]. Moreover, TF and thrombin provide a link between coagulation and inflammation. Endotoxin and inflammatory cytokines induce TF expression on activated monocytes/macrophages [52, 53]. TF is able to activate PAR2, in conjunction with FVIIa and FXa, which is the only PAR that is not activated by thrombin [163-165]. Mouse models have shown that genetic ablation of PAR2, leads to reduction in macrophage mediated inflammation of adipose [166].

There are large prospective studies that have shown the association between inflammation and thrombosis. Out of 47,004 participants in a Danish study, 1387 had an episode of VTE and those with a CRP > 3 mg/L compared with those < 1 mg/L, had a 2.4 – fold increased risk of VTE [167]. In the Atherosclerosis Risk in Communities (ARIC) Study, when adjusted for age, race, and gender, those with a CRP ≥8.55 mg/L, compared with the lowest 90% of CRP values, had a hazard ratio of 2.07 (95% CI 1.47, 2.94) for VTE [168]. This is also reflected by studies in inflammatory conditions that demonstrate an increased risk of thrombosis. There are several studies that have shown a 2 to 3-fold increased thrombotic risk in patients with rheumatoid arthritis, irrespective of age, gender and traditional risk factors for VTE [169-171]. There was a cumulative incidence of VTE of 7.2 % in 30 years for RA patients, compared with 3.5 % in the normal population in Minnesota [171]. This is like the finding in Swedish inflammatory bowel disease patients who were associated with a 3-fold increased risk of VTE compared to the general population [172]. One of the mechanisms by which inflammation, and specifically inflammatory conditions may be associated with an increased thrombotic risk is through microparticles (MP), which will be discussed in more detail in Section 1.2.

1.2 Microparticles

1.2.1 Background

MP are heterogeneous cell membrane derived particles measuring 100 – 1000nm in diameter that are thought to play a role in diverse processes, such as thrombosis and inflammation [173-175]. They form by "budding off" cells undergoing apoptosis or stimulation, and are characterised by a lipid bilayer, which expresses surface markers and encase cytosolic components, such as enzymes, mRNA, micro RNA and transcription factors, all derived from their parent cells [176, 177]. MP can be formed from platelets, endothelial cells, neutrophils and monocytes and retain the cell surface markers of their parent cell, which aids identification. Monocyte derived MP often carry surface TF, and have been shown to be increased in conditions associated with thrombosis, such as malignancy [178, 179], VTE [180, 181] and cardiovascular disease [182].

In 1946, Chargaff and West discovered that not only does platelet free plasma clot, but that its ability to do so was impaired after high speed centrifugation. This showed that that there were components other than platelets in the blood that allowed plasma to clot. MP were first discovered by a Birmingham Haematologist, Dr. Peter Wolf [183]. He was investigating why stored frozen citrated plasma became more procoagulant with time. This had been demonstrated when platelets were incubated with citrated plasma, as increasing the time of incubation prior to calcification shortened the lag time to clot formation [184]. Dr. Wolf summarised it thus [183]:

> The purpose of the present communication is to provide evidence for the occurrence in normal plasma, serum and fractions derived therefrom of coagulant material in minute particulate form, sedimentable by high-speed centrifugation and originating from platelets, but distinguishable from intact platelets. It is suggested that this material, hereafter referred

to as ‘platelet-dust’, is responsible for the phenomena referred to above. (Quoted from Wolf, 1967)

The ‘platelet dust’ he described was subsequently identified as MP. He used differential centrifugation to discover them, a method which remains an integral part in their assessment, although can lead to other problems in their assessment.

1.2.2 Formation of microparticles

The resting eukaryotic cell membrane is a lipid bilayer, with the outer layer consisting of sphingolipid and phosphatidylcholine, whilst the inner layer consists of aminophospholipids, namely phosphatidylserine (PS) and phosphatidylethanolamine. The asymmetrical distribution of phospholipids is maintained by the actions of cell membrane transporters known as ‘flippases’, ‘floppases’ and ‘scramblases’ (Figure 1-3) [185]. This is an active process, with ‘flippases’ and ‘floppases’ requiring ATP to operate, whilst the ‘scramblase’ is a calcium dependent bidirectional membrane transporter [186]. As a result, the appearance of either aminophospholipid on the cell surface leads to increased activity of ‘flippases’ to maintain normal lipid bilayer integrity [187].

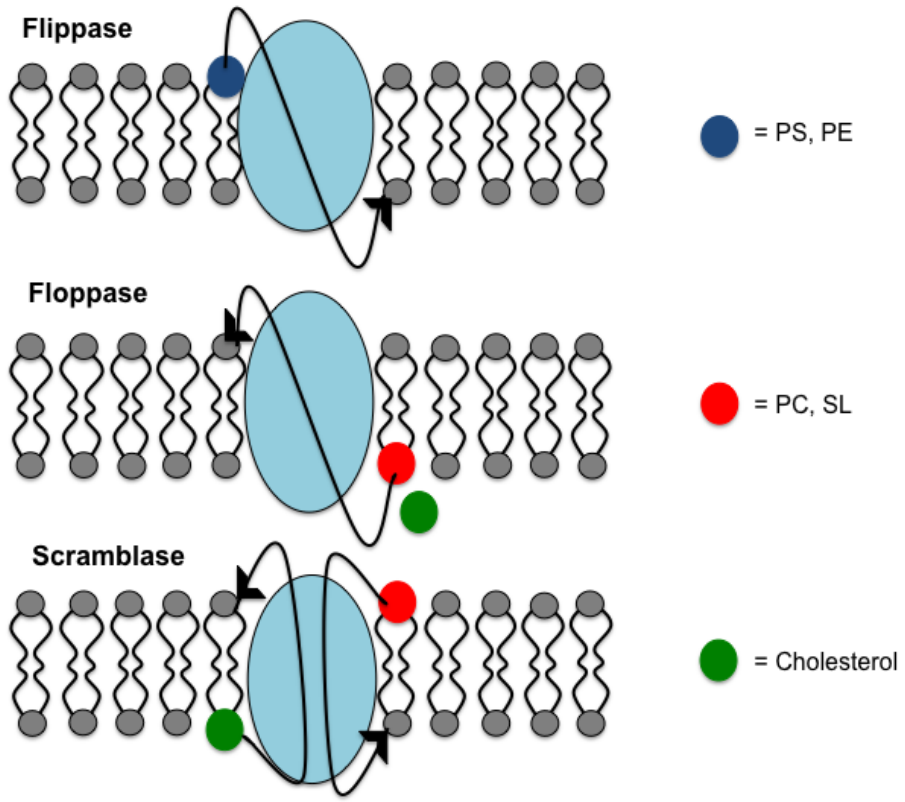
It is thought that ‘floppases’ mediate the rapid outward translocation of aminophospholipids. The Xk-family protein Xkr8 has been found to be responsible for mediating PS exposure in response to apoptotic stimuli and is likely to be a ‘floppase’ [188]. This was confirmed when both mouse Xkr8−/− cells and human cancer cells, in which Xkr8 expression was repressed by hypermethylation, failed to expose PS during apoptosis and so were not consumed by phagocytes [188].

Rapid outward translocation of aminophospholipids is assisted by the action of a ‘scramblase’, which promotes movement of phospholipids down their concentration

gradient in response to a rise in intracellular calcium [189]. A calcium influx inhibits the action of ‘flippases’, leading to the externalisation of PS as a result of the overwhelming actions of both ‘floppases’ and ‘scramblase’ [186, 190]. This results in lipid layer rearrangement and randomisation.

Alongside lipid bilayer rearrangement, proteolysis of calcium-activated calpains is occurring, and this combination of intracellular calcium dependent processes promotes the shedding of MP [191]. Consequently, preferential surface expression of PS is a common feature of MP, suggesting that non-PS expressing MP ought to be a relatively uncommon event [192]. Surface PS expression leads to an anionic surface that provides binding sites for activated coagulation factors [193]. PS is also a useful marker of MP, since it binds annexin V (AnV+) in a calcium dependent fashion, so fluorochrome- conjugated AnV can be used as a marker of MP in flow cytometry [194]. AnV can be utilised alongside cell surface markers to specifically identify cellular origin of MP (Table 1-1).

Figure 1-3 The action of 'flippase', 'floppase' and 'scramblase'



Both 'flippases' and 'floppases' are ATP dependent membrane transporters. 'Flippases' move phosphatidylserine (PS) and phosphatidylethanolamine (PE) inwards, whilst Floppases move phosphatidylcholine (PC), sphingolipid (SL) and cholesterol in the opposite direction. Both act against the concentration gradient. 'Scramblase' are ATP independent and facilitate the movement of lipids along concentration gradients. (From Clark, 2011) [169]

1.2.3 Detection and analysis of microparticles

Since the initial discovery and identification of MP, the methods used to detect them have evolved. However, there remains significant variation in both pre-analytical and analytical variables, with standardisation of protocols currently being approached separately by the haemostasis and extracellular vesicle (EV) research communities [216-218]. Currently, the main pre-analytic variables for consideration include sample acquisition, processing and storage. Analytical variation encompasses the methods used to detect MP, including both instruments and the use of cell surface markers. These markers are summarised in Table 1.1.

Table 1-1 Surface markers to characterise source of microparticles

Source	Marker	Alternative name
Platelets	CD42a CD41 CD62P	GPIX [195, 196] GPIIb [197, 198] P-selectin [199, 200]
Endothelial cells	CD31 CD144 CD105 CD62e CD54 CD106	PECAM-1 [201, 202] VE-cadherin [193, 203] Endoglin [204, 205] E-selectin [204, 206] ICAM [207] VCAM-1 [208]
Neutrophils	CD15 CD66b	Lewis-X, 3-FAL [209] CEACAM8 [210, 211]
Monocytes	CD14 CD142	LPS-R [212, 213] Tissue Factor [214, 215]

1.2.3.1 Sample acquisition and storage

The collection of peripheral blood samples and their storage is subject to several variables. Blood collection method, the anticoagulant, type of sample, such as plasma, serum or whole blood, and storage can all impact on MP number. Subsequently, how the sample is handled, such as number of freeze-thaw cycles can have a considerable impact.

1.2.3.1.1 Collection of the peripheral blood sample

Needles of gauges 18 to 22 are often used when collecting peripheral blood samples for haemostasis testing [219]. Narrower gauge needles are associated with an increased risk of haemolysis and cellular activation than larger gauge needles, because of higher shear stress. In coagulation testing, a haemolysis as low as 0.9% can have a significant impact on coagulation testing results [220]. Furthermore, high

shear stress during blood withdrawal can cause MP formation from haemolysis and platelet aggregation [221, 222]. Commonly, needle gauges 19 to 21 are used for peripheral blood withdrawal, and these gauges are shown to lead to no difference in MP count [223].

To avoid the impact of cell activation, the initial volume of the blood collected is discarded from analysis. Vascular damage occurs during venepuncture leading to increased circulating endothelial cells in the first 1ml of blood collected after venepuncture, compared to the first 1ml of blood collected after discarding the initial 7.5 mls [224]. For this reason, it is recommended in consensus guidelines and various studies that the first 3 – 7.5ml collected is discarded [213, 216, 225-227].

1.2.3.1.2 Choice of anticoagulant

The most commonly used anticoagulant for MP analysis in the literature is citrate [217, 228]. Citrate, like ethylenediaminetetraacetic acid (EDTA), chelates free calcium ions leading to anticoagulation [229]. The stock concentrations of citrate are 3.2% (109 mmol/L) and 3.8% (129 mmol/L). To maintain consistency in coagulation testing, it is recommended to use 3.2% at a 1:9 concentration [230].

There is a variable impact of citrate and EDTA discussed in the literature. Citrate has been shown to prevent degranulation of polymorphonuclear cells that occurs because of dialysis [231, 232]. Moreover, chelating calcium reduces lipid membrane budding and MP formation, which is dependent on calcium [186]. These findings suggest that citrate does not lead to an increase in celluar activation. Both citrate and EDTA, however, have been shown to reduce MP count by adherence of MP to platelets, but EDTA reduces MP more so than citrate [233]. In contrast, EDTA has also been shown to dissociate the GPIIb/IIIa complex and lead to platelet activation,

leading to an increased number of MP [234, 235]. Moreover, EDTA is accompanied by a very high concentration of potassium, likely leading to increased MP formation. Potassium leakage from stored red blood cells has been shown to lead to decreased 'flippase' activity and increased 'scramblase' activity, resulting in increased lipid membrane budding and MP formation [236].

Heparin is not recommended for use in MP flow cytometry. It has been shown to increase MP counts, possibly due to *ex vivo* activation of cells leading to MP production, because extracellular calcium is not being chelated [223, 233]. Like low molecular weight heparin, it aggregates and activates platelets leading to MP formation [217, 237]. Moreover, it has led to a reduction in the threshold of platelet activation [238]. It also stimulates TFPI expression from endothelial cell lines, thereby affecting TFPI measure [91]. It appears that calcium chelating anticoagulants are best, with citrate being a better option that EDTA, although issues remain.

1.2.3.1.3 Issues surrounding sample collection

The time between collection and processing of the sample needs to be short, but the temperature at which this is done does not seem to have an impact. It is recommended that samples are processed within 1 – 2 hours of collection and that there is little agitation of the sample after collection [239, 240]. When whole blood is stored, there is increasing cell membrane budding, leading to a rise in MP. The same is true of agitation, which is why some recommend keeping samples vertical [236].

Dyslipidaemia is also important to consider. This results in increased levels of oxidised low density lipoprotein (LDL), which has been shown to increase TF

expression in THP-1 monocyte cell lines and human monocytes, increasing the release of TF+ MP [241]. This suggests that lipid-lowering therapy would have an impact on MP measure. Atorvastatin has been shown to decrease thrombin generation and TF, GPIIIa and CD62P expression on platelet-derived MP, which appears to be due to a reduction lipoprotein levels thereby reducing MP formation from cell activation [199]. Other statins have been shown to decrease thrombin generation by inhibiting TF expression by monocytes [242, 243]. Fasting prior to blood collection is routinely used to minimise LDL, however there are no studies investigating the impact of this on MP formation. Also, it is unclear what the impact of statin therapy is on MP release in patients with normal lipid levels, to see whether there is an additional effect beyond reducing lipoproteins. Practically, it may be difficult for patients to fast before giving samples, so it is routinely recommended in MP analysis [218, 239].

1.2.3.2 Plasma or serum

Over an extended period, citrated blood becomes more procoagulant [184]. Therefore, peripheral blood samples require careful handling and processing to avoid cellular activation that can impact negatively on sample assessment. The majority of studies use platelet poor plasma (PPP), and guidelines from the EV and haemostasis communities recommend assessment using plasma [218, 244]. Serum is not recommended as a source to identify MP. George in 1982 compared the amount of platelet-derived MP in plasma to that in serum using immunoelectrophoresis with an antibody against glycoprotein IIb/IIIa and found that MP concentration was ten times higher in serum than plasma samples [245].

There are numerous groups that have assessed MP from whole blood [246-248]. The main advantage of whole blood samples is that the absolute number of MP can

be measured, because there is less processing. This is due to there being no centrifugation steps [248]. PPP centrifuged at 10,000g for 10 minutes led to a significant decrease in CD31+ CD42b- endothelial cell derived MP [227]. A decrease in MP has been shown when spinning PPP at 13,000g for 2 minutes of 80%; and also, a 52% decline in TF activity compared to that expected [249, 250]. This suggests that MP declines rapidly with high-speed centrifugation.

There are three key issues with whole blood analysis when doing flow cytometry: inability to batch process, background noise and difficulty in using fluorescent labelled antibodies. Whole blood samples should be run quickly following collection to avoid increased platelet activation and MP formation [251]. This occurs despite the MP being in their normal milieu. The temperature at which samples are processed at is also a consideration, as FVIIa and FVIII levels are known to rise when citrated blood is cooled [252]. Therefore, many guidelines recommend that samples are initially processed at room temperature [216, 218, 253]. The need to process whole blood almost immediately to accurately assess MP prevents batch processing and limits the number of samples that can be assessed at a time.

PPP has several advantages over whole blood when analysing MP, so many groups use PPP in preference to whole blood. The most frequent reason PPP is used is to avoid platelet activation that generates further MP [217]. The majority of MP are derived from platelets (up to 70-90%), so platelet activation will cause a substantial rise in MP, resulting in misleading results [193, 254].

There are various methodologies used to derive PPP in the literature [204, 218, 239, 250, 255]. Ultracentrifugation ought to be avoided, because of increased shear effect and platelet aggregation, which can increase the amount of MP produced [221]. Some groups use ultracentrifugation together with a sucrose density gradient that allows buffeting of the MP, and separation of MP according to molecular weight

[256, 257]. This technique is most used when analysing for exosomes, rather than MP, and requires extensive processing to separate them from the sucrose gradient [256]. This is onerous, so the technique is not commonly used.

Once PPP samples are collected they can be stored at -80˚C, which allows batch processing of samples. This is an advantage over whole blood samples. Some groups advocate snap freezing in liquid nitrogen prior to storing at -80˚C, which seems to make little difference in MP recovery, unless samples are thawed on ice, when there is a reduction [258]. Freeze-thaw cycles are generally avoided as confirmed in guidelines [216, 218, 259], although there is a report showing that freeze-thaw has no impact on MP, particularly those of smaller sizes [233].

There are several disadvantages with PPP. Many of the cell stains used for MP - analysis require a calcium-containing buffer to allow binding, such as AnV binding to surface PS. This will lead to clotting of samples, and although heparin can be used, this leads to further dilution of samples, and, as noted previously, heparin also leads to increased TFPI expression in circulating cells [91]. There remains significant background noise when using PPP, which impacts on analysis. This, however, depends on the modality used. For example, in flow cytometry, PPP or a MP suspension can be used, whilst in nanoparticle tracking analysis, PPP is the preferred sample type**.**

An MP suspension is invariably derived from PPP, so has the advantage of being stored at -80˚C as PPP prior to use. The main advantage is that the PPP undergoes an additional centrifugation step to sediment MP, after which the rest of the plasma is discarded and the MP pellet is resuspended in the appropriate buffer. This has the advantage of higher sample purity, which leads much less background noise. It also allows easier use of various cell membrane stains containing calcium, since there is so little plasma in the sample, there is a reduced risk of clotting.

Overall, although there are several disadvantages with MP suspensions, the main advantage is being able to use a variety of different stains with MP suspensions, and the reduced risk of coagulation. They can also be added to functional assays such as thrombin generation or TF assays, which may require the addition of standard plasma without contamination, thereby maintaining consistency from the functional assays to flow cytometry.

1.2.4 Assessment types

Assessment of MP tends to fall into three types: enumeration and cell surface characterisation, anatomical and function. None are used in isolation, and a combination is often required. The major modalities used to assess MP are as follows.

1.2.4.1 Flow cytometry

1.2.4.1.1 Background

Despite the availability of new technologies, flow cytometry remains the most extensively utilised technique used to enumerate and characterise MP in the literature [201, 211, 214, 260-263]. This is probably the result of its accessibility, as it has been present and used in many fields of biological science for over 50 years [264]. The focus of this section is the application of flow cytometry in the analysis of MP.

In flow cytometry, particles are suspended in a stream of fluid that passes through a flow cell and interacts with a laser beam. This interaction allows enumeration, sizing and assessment of surface characteristics of particles to take place, at a rate of over a thousand particles a second.

All particles scatter light, and these can be measured by forward and side detectors. Forward scatter light is measured at 1 - 20˚ to the incident light, and is proportional to the square radius of the particle allowing measure of the size [265-267]. In flow cytometry, the shortest wavelength of the light is 488 nm, so the sizing of MP by forward scatter is often inaccurate, and can differ significantly from that of silicon or latex microbeads [216, 268].

Side scatter of light is measured perpendicular to the incident light, at any angle between 30 and 150˚, but often at 90˚. It gives information on granularity and can also inform on size. This granularity can be affected by cytoplasm, so there are differences between latex microbeads and biological cells [216, 268]. Several groups have shown greater discrimination between particles in side scatter, which may be due to some flow cytometers having a smaller forward scatter collection, compensating in side scatter [269, 270]. This has led to the development of recommendations on assessing MP in both forward and side scatter, but differences between flow cytometers need to be considered [271]. The role of forward and side scatter is to define the upper limit of MP sizing using 1 μm microbeads, whilst the lower limit remains difficult to define and is subject to the machine's background noise, which comes from the fluidics and electronics [244].

1.2.4.1.2 Microparticle detection

There are several groups that use no cell surface markers, and use only forward and side scatter. This can be problematic, due to the physics of scatter from tiny particles, and the risk of detecting particles that are not MP, such as fluorescent protein aggregates [272] and lipoproteins [218]. To better identify MP, cell surface markers such as AnV and biomaleimide are often used [207, 268, 273, 274] [275, 276].

AnV is the most commonly used cell surface marker for MP. It is a 35 kDa polypeptide chain that forms calcium-dependent units when encountering PS [194]. It can be conjugated to many different fluorochromes, and is often used to identify apoptotic bodies [277]. Not all MP bind AnV, suggesting there are several MP that are not expressing PS, and the binding of AnV is calcium-dependent [198, 278].

Biomaleimide binds to cysteine residues and thiol groups in proteins within cellular membranes, and is fluorescent so can be used in flow cytometry [275]. It stains all cell membranes, so does not differentiate between those that have a rearranged lipid bilayer membrane and those that do not. The staining is rapid and there is no washing step required before analysis [279]. The main concern with biomaleimide is that it detects all particles, including several particles that are not MP, so rigorous sample preparation is vital to prevent inclusion of a considerable number of lipoproteins.

A concern when measuring MP by flow cytometry is whether they are detected as single events or as a ‘swarm’. The swarm consists of smaller MP, which aggregate and leads to an underestimate of the number of smaller MP, whilst there is an overestimate of the larger MP [280]. Dilutions are often performed to assess this, but there are other variables to consider, such as the initial ‘boost’ and by slowing the event rate, which reduces the risk of swarming [218, 281, 282]. This remains a constant concern and groups recommend not concentrating MP, and measuring at a slow rate to prevent aggregation of sample.

1.2.4.1.3 Antibodies and fluorochromes

Any antibody can be used to try and identify MP, but the types and number may vary with cellular origin, and often mirror that which occurs with cellular flow

cytometry. For example, when performing platelet MP flow cytometry, standard platelet surface markers such as CD41a, CD41b, CD42a and CD42b are used, with CD62P used to identify activated platelets [201, 216, 283-285]. When studying endothelial cell-derived MP, multiple markers have been used such as CD31 and CD105, but in order to discriminate them from platelet-derived MP (which also express CD31 and CD105), a platelet marker must also be used to ensure this is negative[202, 206, 286-288]. There are a number of groups that use CD144, which is a junctional protein specific for endothelial cells [193, 203]. It is also important to use negative isotype controls and make sure that these are well matched [244]. This is to make sure that there is no non-specific binding and the signal is truly the particle of interest.

1.2.4.1.4 Background noise

Background noise can come from the fluidics, optics and machinery, including electronic noise from lasers [244]. In many flow cytometers there is a 0.22 μm filter that filters out much of the sheath fluid, but it still important to use filtered sheath fluid to reduce the background noise [279, 289]. It is also important to keep this free of fungus with the use of a detergent.

The main issue described with flow cytometry is optical noise. This can occur with auto-fluorescing dust particles, which in normal cell-based flow cytometry are removed by thresholding so that only particles larger than a specified size, such as 1 – 2 μm in diameter, are included in the analysis. This has the added benefit of reducing file sizes. This is not possible in MP flow cytometry, where we are aiming to have the very smallest particles included in the analysis. It is important to make sure that there is regular cleaning of the flow cytometer before and after use, using filtered cleaning reagents and to make sure that all buffers are filtered.

Whilst flow cytometry is not without issues, its accessibility, ability to batch process and the amount of data that can be garnered means it remains the most commonly used modality. There is also greater experience with this technique than others, and it remains the technique that is leading standards. However, consideration should also be given to alternative modalities.

1.2.4.2 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) detects particles by Brownian motion using light scattering [290]. There is a reciprocal relationship between the size of the particle and speed of movement. Unlike other dynamic light scattering imaging techniques, it is able to detect smaller particles of up to 70 -90 nm particles consistently, whilst flow cytometers can only detect particles > 300nm in diameter [291-293]. There are however several issues with this technique. Firstly, this technique involves dynamic light scattering and is dependent on the camera which can have difficulty resolving light from particles that may be 5-fold different in size [257]. Although fluorochrome conjugated antibodies can be used, the majority of groups use this technique to identify total numbers of MP or other EV [217, 290, 294, 295]. The main concern is that this can lead to an overestimate in MP numbers, because size is the only discriminator. It is known that lipoproteins and chylomicrons can be confused for MP, so it is important that samples are taken from fasting patients [218]. Rather than being the primary method to analyse for MP, it is often used as an adjunct, such as confirming counts after a population of MP has been sorted by flow cytometry.

1.2.4.3 Functional assays

Functional assays tend to focus on the pro-coagulant capability of MP, and amongst the assays studied are generation assays. Thrombin generation assays have been available for several decades, and model the thrombin produced as a clot forms, and can measure the impact of various clot factors, anticoagulants and antiplatelet agents [296-298]. Kinetic modelling of thrombin generation has been achieved using the calibrated automated thrombin generation (CAT) assay, using a fluorogenic substrate that reacts slowly, thereby permitting the assessment of thrombin generation with time [299]. There are several different measures including time to thrombin generation, peak thrombin and endogenous thrombin potential (ETP) [300]. The ETP is the amount of activity and is proportional to the concentration of thrombin.

There are several groups that have shown the efficacy of the CAT assay in identifying the procoagulant activity of MP. Thrombin generation has been shown to be greater in patients with thrombosis, especially those with recurrent thrombosis [301]. Increased thrombin generation and reduced lag time to thrombin generation has been demonstrated in plasma from prostate cancer patients [302]. Enhanced thrombin generation has also been found in child patients with vasculitis as a result of MP isolated from plasma samples [303].

Mackman's group predominantly analyse MP by a combination of in-house FXa generation, thrombin-antithrombin and MP TF activity assays [178, 304-307]. These have been shown to successfully associate increased coagulant activity and the increased presence of MP, particularly TF+ MP. The Zymuphen MP-TF kit is a commercial ELISA, that captures MP by a non-neutralizing monoclonal antibody (mAb) to TF [308]. It has been shown to identify increased MP-TF activity in plasma

from patients with obstructive sleep apnoea and myeloproliferative neoplasms [309, 310].

1.2.4.4 Other techniques

Western blotting has been used in MP analysis, but as flow cytometry has become more ubiquitous and newer techniques been developed, it has become less common. Its main role in EV research is in the analysis of exosomes. It is useful in identifying exosomes by surface tetraspanins (CD9, CD63, CD81, CD82) and detecting proteins in exosomes isolated following sucrose density centrifugation [218]. Transmission electron microscopy (TEM) and atomic force microscopy (AFM) have also been used to assess MP, but are not as commonly as other techniques. TEM can detect the smallest particles because the wavelength of the electron is smaller than light, so it is able to resolve any EV with high resolution [311]. For that reason, it is a very good technique for analysing the morphology of single adhered MP, but is unable to quantify MP. Sample preparation is onerous and leads to dehydration of particles, so size measurement is often inaccurate using TEM [217, 312].

AFM is a newer imaging technique, which is able to show the topography of MP by measuring the force between the probe and the sample [313]. The lateral resolution is approximately 30 nm, whilst the up and down resolution is 0.1 nm, so it can provide a large amount of detail at high resolution [313]. A major issue with AFM is sample preparation, which is the same as that with TEM. As with TEM, it is labour intensive, and it gives detailed information about one particle, but is unable to general information on numerous particles and enumerate them [314].

1.2.5 Microparticles and thrombosis

There is increasing acknowledgement that MP play a role in thrombosis [182, 315, 316]. This has particularly been recognised in cancer patients with a history of thrombosis. TF+ MP have been detected in two-thirds of advanced pancreatic cancer patients, a tumour type that is particularly associated with VTE [315]. Pancreatic and metastatic breast cancer patients with prominent numbers of TF+ MP and increased TF activity were more likely to develop thrombosis on 12-month follow-up than those without [317]. In this cohort, MP levels dropped in three patients who underwent pancreatectomy, suggesting that the TF+ MP were mostly tumour derived. Increased TF+ MP activity has also been reported in patients with metastatic breast cancer compared to healthy controls, but the same increase was not found in those with non-metastatic breast cancer [315, 317].

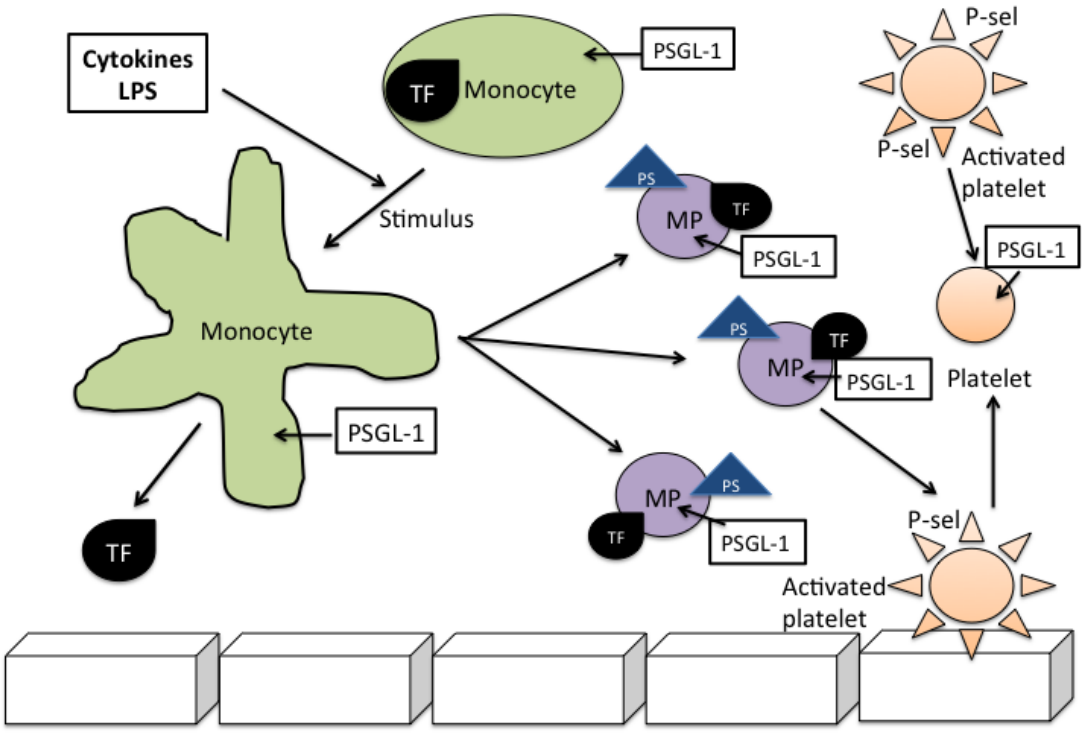
It is unclear whether the level of TF+ MP is a marker for thrombotic risk in cancer patients. In colorectal cancer patient cohort, a two-fold higher level of TF+ MP correlated with an increase in d-dimer and increased risk of thrombosis [318]. Conversely, TF+ MP levels were no different in 67 women with an ovarian malignancy compared with 42 women without, and there was no correlation between d-dimer and TF+ MP numbers [274]. 42 women control group contained 18 with uterine fibroids, which may be a confounder, because an increased risk of thrombosis has been reported in patients with uterine fibroids [319].

In a retrospective analysis of 60 cancer patients initially without VTE, the cumulative incidence of VTE was 38% at 12 months for those with increased level of TF+ MP and 0% for those with a low level [315, 320]. The outcome of that study suggested that TF+ MP could be used as a biomarker to assess thrombotic risk in cancer. Subsequently, a prospective placebo-controlled study was undertaken to assess the efficacy of enoxaparin as thromboprophylaxis in patients with high TF+ MP in

advanced cancer. Enoxaparin was shown to reduce the risk of VTE in these patients with a high level of TF+ MP at 2 months, from 27.3% to 5.6% but this difference was not significant (p = 0.06) [179].

TF+ MP have been shown to play a significant role in thrombus propagation in murine models. TF+ MP were shown to possibly be the only source of TF within the growing thrombus, although the presence of TF+ MP was dependent on P-selectin glycoprotein ligand -1 (PSGL-1) (Figure 1-4) [321]. PSGL-1 is also expressed on CD14+ MP and tumour cell derived MP, and it binds to P-selectin (CD62P), which is expressed by activated platelets and endothelial cells [322]. This interaction between CD62P and PSGL-1 allows the accumulation of TF+ MP in the thrombus.

Figure 1-4 Propagation of thrombus dependent on PSGL-1



A schematic of tissue factor (TF) bearing and monocyte-derived MP (TF+ MP), also expressing P-selectin glycoprotein ligand-1 (PSGL-1), which are preferentially expressed by these MP and bind to P-selectin (P-sel) on activated platelets, leading to accumulation of TF+ MP within the propagating thrombus, thereby denoting the essential role of PSGL-1 and P-sel in thrombus propagation. (From Furie, 2008) [610]

It remains controversial as to whether blood-borne tissue factor is present and is functional [321, 323-325]. Immunostaining has confirmed the presence of TF-containing neutrophils and monocytes in the circulation [323]. Yet the TF-related activity in the blood is low [325]. This suggests that much of the TF in the blood is latent, and is only active in areas of inflammation, so that it encounters fibrin or neutrophil extracellular traps [326]. On activation, platelets and endothelial cells release thiol isomerases, including protein disulphide isomerase (PDI), which catalyses disulphide oxidation, reduction and isomerization, thereby playing an important role in protein synthesis [327]. It appears that the action of this enzyme is required to switch TF from latent to active form, thereby controlling TF procoagulant activity [328].

The interaction between PSGL-1, CD62P and TF suggests that PSGL-1 may be a viable target for therapy to prevent thrombosis. In a baboon model, 12 baboons had IVC thrombosis induced following IVC balloon occlusion, 8 of who were pre-treated with recombinant -PSGL-1 immunoglobulin. Those who had been pre-treated demonstrated a significant inhibition of thrombosis and venous wall inflammation, as well as a reduction in IL-8 and MCP-1 levels [329]. Notably, these cytokines are raised in inflammatory conditions, such as Behçet's syndrome [330, 331].

An association between circulating levels of MP and idiopathic VTE has also been described. In a case-control study of 186 patients diagnosed with their first VTE, versus 418 healthy controls, the risk of VTE increased in a dose-response manner with the level of MP [181]. This risk of VTE was independent of age, gender, presence of hereditary thrombophilia, body mass index and factor VIII levels. The level of MP may also vary with the acuity of the thrombotic event. In one study, all patients had an increased MP count than healthy controls, but the level was lower in those with a more recent thrombosis than those who had one in the past [332]. This

may be reflective of increased consumption of these MP in the thrombus of patients with an acute episode.

1.2.6 Microparticles in cardiovascular disease

Atherosclerosis is a chronic inflammatory state characterised by increased endothelial cell permeability and the accumulation of low-density lipoproteins in the subendothelial space; followed by the diapedesis of leukocytes and formation of foam cells, migration and proliferation of smooth muscle cells, production of connective tissue and neovascularization [333]. Ultimately, this leads to plaque rupture and vessel occlusion causing ischaemia and cell death. Even in patients with subclinical atherosclerosis, as shown with ultrasound of the abdominal aorta, femoral arteries and carotid arteries, were found to have MP [205]. Moreover, patients following coronary artery bypass grafting were shown to produce MP that promoted thrombin generation in a TF-dependent manner [334].

1.2.7 Rheumatoid arthritis

In rheumatoid arthritis (RA) numbers of MP rise as disease activity scores increase [283]. They have been found in synovial fluid of inflamed joints and promote coagulation via the TF-dependent pathway. TF+ MP are also present and support more rapid fibrin deposition, a denser fibrin network than platelet-derived MP, and are likely to cause fibrin deposition in synovial fluid [262, 335]. MP bound C1q, C4 and/or C3 tend to be more abundant in RA synovial fluid, correlating with synovial fluid IgM and IgG levels [261]. This is present early in the course of RA, but does not significantly decline with immunomodulatory therapy, suggesting that MP are not directly associated with inflammation, but are more specific to the RA disease process

[336]. Synovial fluid MP act as signalling molecules and signal the release of B-lymphocyte regulators, such as B-cell activating factor. They also increase the levels of monocyte chemoattractant protein-1 (MCP-1), IL-8, RANTES and vascular endothelial growth factor (VEGF) when incubated with synoviocytes, all of which have been implicated in the inflammatory process of RA [337, 338].

1.2.8 Systemic lupus erythematosus, antiphospholipid syndrome and connective tissue disorders

There is conflicting evidence of the role of MP in connective tissue disorders. Increased circulating platelet-leucocyte complexes and platelet activation, assessed by measuring CD62P were found in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS), but not raised platelet-derived MP [339]. Endothelial MP have been shown to be increased in patients with SLE, particularly those with antiphospholipid antibodies, suggesting that they are associated with a procoagulant phenotype [206]. Another study has shown that AnV+ MP were lower than healthy controls, in particular leucocyte- and platelet-derived MP populations, whilst AnV- MP populations were found to be associated with hypertriglyceridaemia, nephritis and more severe disease [273]. A confounder is the presence of chylomicrons, which may possibly have been assessed as part of the AnV- MP population, especially since the peripheral blood samples were not fasting. Platelet-derived MP are increased in SLE patients compared to healthy controls, but little difference was found between active and inactive disease, and no study has focussed on those with a thrombotic phenotype [284].

Platelet-derived MP are raised in systemic sclerosis and mixed connective tissue disorder compared to healthy controls and this correlates positively with Raynaud's phenomenon [340]. There is an increased number of endothelial-cell derived MP

populations (CD62E+, CD31+, αvβ3+ MP) in lupus anticoagulant patients with thrombosis than those without [206]. Variability can be within the disease, as exemplified by those with systemic sclerosis. Here, MP were found to increase with extent of systemic sclerosis (higher in diffuse than limited), whilst it declined with increase in modified Rodnan skin score. The discriminator appeared to be a score of 10/51, and the presence of oral ulcers [341]. This is a little difficult to reconcile because the modified Rodnan skin score correlates well with skin biopsy weight and extent of scleroderma [342].

1.2.9 Vasculitis

Brogan and colleagues have been investigating MP in childhood vasculitis. Endothelial cell derived MP are found to be elevated in childhood vasculitis patients, where they rise with activity and decline with successful therapy, suggesting a role as a biomarker [203]. Data from the same group showed that thromboembolic disease in childhood vasculitis patients is associated with MP mediated thrombin generation [343]. Herein they showed that those with active vasculitis, including polyarteritis nodosa, granulomatous polyarteritis and BS, generated higher peak thrombin as measured with the CAT assay, and this correlated with the total number of MP [300]. Anti-neutrophil cytoplasmic antibodies (ANCA) are known to stimulate the release of neutrophil derived MP, compared with healthy controls and those with inactive vasculitis, in particular CD11b+ rather than CD66b+ neutrophil derived MP [344]. However, CD11b is present on other leucocytes, whilst CD66b is more specific as a marker of neutrophils [345].

1.2.10 Behçet's Syndrome

In Behçet's syndrome (BS) there have been few studies on MP. In the Brogan study, there was only one patient with BS who also had cerebral vein thrombus. He had elevated levels of total MP and peak thrombin, consistent with the other patients with active vasculitis [343]. Platelet and neutrophil activation, including levels of MP have been investigated in BS patients. 43 BS patients, fulfilling the 1990 ISG criteria were studied and it was found that CD42a+ CD62P+ MP were increased in BS patients compared to healthy controls [346]. In this paper, MP were identified by size and there was no additional membrane dye or AnV to discriminate further. A more recent study of 55 BS patients, 29 men and 26 women, assessed MP by function using a capture ELISA, with wells coated with AnV to bind to surface PS [347]. This showed a significantly increased level of procoagulant activity secondary to MP, but this was not a discriminator for clinical characteristics and thrombotic risk in BS. As with many other studies, functional analysis of BS samples, in particular aimed at identifying those at increased risk was not effective. This thesis focuses on the role of MP in BS, and therefore BS will be discussed in more detail in Sections 1.5.

1.3 Behçet's Syndrome

1.3.1 History

Behçet's syndrome (BS) is a chronic multisystem inflammatory disorder of unknown aetiology, characterised by recurrent oral aphthae, genital ulcers, uveitis and skin lesions [348]. The first known description of a condition thought to be BS was by Hippocrates in the 5th century BC [349]. An English translation of his description reads:

> There were other fevers also……. Many had aphthae and sores in the mouth. Fluxes about the genitals were copious; sores, tumours external and internal; the swellings which appear in the groin. Watery inflammations of the eyes, chronic and painful. Growths on the eyelids, external and internal, in many cases destroying the sight, which are called "figs". There were also often growths on other sores, particularly in the genitals. Many carbuncles in the summer, and other affections called 'rot'. Large pustules. Many had large tetters [skin disease causing pruritic pustules or patches]. (Quoted from Hippocrates, 5th Century)

In this passage, Hippocrates is documenting the key manifestations of BS: oral aphthosis, genital ulceration and eye inflammation. Hippocrates also describes 'figs' or growths, which are not normally part of a diagnosis of BS. However, a case of lacrimal gland hyperplasia in a patient with recurrent oral aphthosis, pustulosis and inferior vena cava thrombosis has been reported [350].

Clinical reports of entities similar to BS have been published since the 18th century, with reports from Reiss (1906), Blüthe (1908) and Gilbert (1925) in the early 20th century. These all document cases consisting of iridocyclitis, mucocutaneous lesions and arthritis that would fulfil a modern diagnosis of BS [351]. Subsequently, there was increasing recognition that this constellation of symptoms and signs represented a single disease entity or syndrome. By the 1930s and 1940s, there were original descriptions of patients with these manifestations by Benediktos

Adamantiades and Hulusi Behçet that enabled the discovery of this clinical syndrome.

Benediktos Adamantiades (1875 -1962) was a Greek Ophthalmologist who presented a lecture titled ‘A case of relapsing iritis with hypopyon’, during the annual meeting of the Medical Association of Athens November 15, 1930 [351]. The lecture was based on a case of a 20-year-old male patient who initially presented with recurrent oral aphthosis and thrombophlebitis and subsequently he developed recurrent iritis with hypopyon and sterile arthritis, fulfilling a modern diagnosis of BS. This lecture was published in *Proceedings of the Medical Society of Athens* in 1930 [352]. In the following year, Adamantiades connected the presence of genital ulcers, arthritis and ocular lesions as signs of a single syndrome and presented this in the French journal Annales d'Oculistique [353]. In 1946 Adamantiades reported on two further patients and defined thrombophlebitis as the fourth cardinal sign of this syndrome [354].

Dr. Hulusi Behçet (1889 – 1948) was a Turkish Dermatologist who described three patients that he had followed for years [355, 356]. The symptoms originally described by Behçet constituted a triad: aphthous stomatitis, genital ulceration and recurrent pustulosis that he originally published in an article in Dermatologische Wochenschrift in 1937. Behçet went further than Adamantiades in specifically ascribing additional symptoms such as periodontitis, jaw cysts, acneiform skin lesions and erythema nodosum to a ‘triple symptom complex’ in articles in 1940 and 1941 [351].

> First transient aphthous changes in the mouth, secondly ulcerations on the genitalia, and thirdly attacks of iritis, although the latter symptom is not always present. (Quoted from Behçet, 1940)

Behçet is describing the episodic and recurrent nature of symptoms that has remained an important aspect of diagnostic criteria ever since. Tage Jensen (1941) was the first clinician to use the term ‘Behçet’s syndrome’ and this term continued to be used through the 1940s and 1950s [357, 358]. It has remained the term of choice, although there are a number of articles that continue to recognise the contribution of Adamantiades in the discovery of this syndrome [351, 359-361]. However, as of August 2015, a PubMed search reveals 8487 articles with the keywords ‘Behçet’ and 137 with ‘Adamantiades-Behçet’.

1.3.2 Epidemiology

BS is predominantly a disease of the ‘Old Silk Route’ that stretches from the Mediterranean basin, through Iraq and Iran, into Japan and South-East Asia, as shown in Figure 1-5. This suggests that causative factors, including several genetic predisposing factors such as HLA-B51, may be more prevalent along this route [362]. BS appears to be more common in the Mediterranean, with the highest rates recorded in Turkey [363]. Reported prevalence rates are shown in Table 1-2. Prevalence rates must be interpreted with caution between studies however, as there are multiple diagnostic criteria that have been used, making direct comparisons difficult.

Figure 1-5 Old Silk Route



It is likely that there are additional epigenetic and environmental risk factors that impact on the prevalence of BS. This is exemplified by West Berlin, where the frequency of BS had increased from 1.68 to 2.26/100,000 between 1989 and 1994 with Turkish immigration [361]. However, the prevalence of BS in the immigrant Turkish population in Germany remained lower than that in Turkey. An increase in BS cases was also noted in post-war Japan, although the increased awareness of BS as a condition may have contributed to this [364]. Additionally, prevalence studies are complicated by the heterogeneity in the presentation of BS, with frequency of manifestations varying not only between endemic and non-endemic regions, but also along the 'Old Silk Route'. This is demonstrated by gastrointestinal disease, which is common in the Far East but less often seen in Turkey [362].

Table 1-2 Prevalence of Behçet's Syndrome

Country	Prevalence/100 000 of population
Turkey	80-420 [363, 365]
Iran	68 [366]
Saudi Arabia	20 [367]
China	14 [368]
Germany	2.26 [361]

1.3.3 Pathological basis

It is now clear that the main microscopic finding at most sites of active BS is immune-mediated occlusive vasculitis. At the cellular level, CD4+ T cells are found in the perivascular inflammatory exudates, and T_h1 cells respond to various stimuli to produce interleukin (IL)-2, interferon-γ, and tumour necrosis factor-β (TNFβ) [369].

In BS, biopsies confirm vasculitis proximal to most lesions, such as ulcers, epididymitis and retinal lesions, with the demonstration of neutrophils adhering to endothelial cells and their subsequent migration into inflamed areas [370]. Thus, vasculitis is the pathologic hallmark of the disease, yet with its predilection for venous involvement, BS does not neatly fit in the vasculitic spectrum.

BS has a complex polygenetic basis, and encounters with various unknown antigens trigger the underlying inflammatory response, as is reflected by the heterogeneous presentation. Debate has focussed on whether BS is a spondyloarthropathy, autoimmune or autoinflammatory condition. Genome wide association study hits have included ERAP1 and links with IL-12/23 [371-375],

suggestive of an association with spondyloarthropathies, however there is no evidence of the sacroiliitis that occurs in such conditions [358, 376].

Autoinflammatory conditions include periodic fever syndromes, such as Familial Mediterranean Fever and TNF receptor associated periodic syndrome, and tend to have equal male and female preponderance. They are characterised by episodic bursts of inflammation, which are often self-limiting and are triggered by the innate immune system [377]. In contrast, autoimmune conditions are characterised by female preponderance, persistent inflammation, antigen specific T- cell activation and autoantibodies. Autoimmune conditions have an association with HLA class II, which is lacking in autoinflammatory conditions. BS shares many similar features with autoinflammatory conditions, but does not feature fevers, nor does it occur as commonly in childhood.

It is possible that BS is in a similar grouping to gout, Crohn's disease and adult onset Still's, which tend to be episodic in nature and have autoinflammatory features, but not characterised by fever [378]. As with gout, colchicine has been shown to be effective in treating BS, particularly mild mucocutaneous ulceration and arthritis [379, 380]. At low concentration colchicine arrests the growth of microtubules, and is known to inhibit neutrophil function, such as chemotactic response and IL-1 release [379]. IL-1 is an important cytokine in autoinflammatory conditions and is amongst a number of cytokines raised in BS, including IL-4, IL-6, IL-8, IL-10 and TNF-α [381, 382]. Recently it has been shown that CXCL-10, which is a chemokine that recruits mononuclear cells, is overexpressed by monocytes upon IFN-ɣ stimulation, possibly contributing to the exaggerated immune response [383].

1.3.4 Diagnostic criteria

The main problem in developing diagnostic criteria remains the unknown aetiology of BS. It is unclear whether there is an infective trigger or whether BS is a spondyloarthropathy, autoimmune condition or an autoinflammatory conditions. Therefore, diagnostic criteria have been set up following the Hippocratic method of grouping together observations to make a symptom complex. The first such attempt was by Curth in 1946 [384]. He focussed on the four cardinal manifestations of BS as described by Hulusi Behçet, of oral ulceration, genital ulceration, ophthalmological manifestations and skin manifestations, including pathergy.

By 1990, there were five sets of BS diagnostic criteria in use. These varied greatly both in focus, and geographically. Mason and Barnes set out criteria in 1969 [358]. This was based on a case series of 33 patients, 25 of who were thought to have definite BS. Of these, 19 of 25 (76%) had arthritis, with the majority having greater than 5 joints involved and a clear inflammatory rhythm to their joint symptoms. This is much higher than many reported series. The proposed diagnostic criteria were as shown in the table below:

Table 1-3 Proposed major and minor criteria

Major	Buccal ulceration Genital Ulceration Eye Lesions Skin lesions	3 major or 2 major and 2 minor required for diagnosis *(Adapted from Mason and Barnes, 1969)[358]*
Minor	Gastrointestinal lesions Thrombophlebitis Cardiovascular lesions	Arthritis Central nervous system Family history

However, consistent with the times and diagnostic criteria in other conditions, these were set up on the basis on small patient sets with significant selection bias, and on eminence rather than evidence. An International Study Group gathered at the 3rd International Behçet's Disease Conference with the intention of standardising the classification through new diagnostic criteria, and reported their new criteria in 1990. The diagnosis of BS is confirmed if a patient has recurrent oral ulceration plus any two of recurrent genital ulceration, skin lesions, eye lesions and a positive pathergy test [385]. These criteria are sensitive, but are particularly specific in comparison with previous diagnostic criteria, measuring 96% in the 60% training sample, and 95% in the 40% test sample. Importantly, they ascribed that oral ulcers were *condition sine qua non* for a diagnosis of BS [386].

A further set of diagnostic criteria were published in 2011 to attempt to increased diagnostic sensitivity. They removed the necessity for oral ulceration to be present, and for pathergy testing to be undertaken; and added in vascular manifestations [387]. It is possible that by removing oral ulceration as a central diagnostic criteria, many conditions could be misclassified as BS [388] [349].

In Japan, there have been separate but concordant sets of diagnostic criteria since the mid-1970s. The Behçet's Disease Research Committee of Japan developed guidelines in 1987 which, together with the subsequent set were seen as overly-sensitive as diagnoses of BS rose from 11,890 patients in 1989 to 16,640 in 1998. Newer criteria in 2003 sought to address these problems. The major characteristics of the revised criteria 2003 are as follows [364]:

1. The main symptoms include recurrent aphthous ulcers on the oral mucosa, skin lesions, ocular lesions and genital ulcers.

2. Additional symptoms are defined as: arthritis, epididymitis, gastrointestinal lesions, vascular lesions, and CNS lesions.
3. The disease types are defined as: complete type, incomplete types, BS suspected, and special lesions. A patient having some of the main symptoms, but not fulfilling the criteria for the incomplete type, is categorized as “BS suspected”.
4. The revised criteria employ clinical and laboratory data for the diagnosis. They include pathergy test and prick test for dead Streptococcus. HLA-B51 and pathological findings of erythema nodosum are also important.
5. The revised criteria list major diseases for the differential diagnosis

Of particular note, they had four separate categories of BS: complete type, incomplete type, BS suspected and special organ lesions. This segregation was dseigned to aid patient identification, thereby improving specificity, and also for classification in future studies. The latency between the onset of symptoms and diagnosis can vary from 5 – 11 years, with no difference between male and female patients [389].

1.3.5 Diagnosis of Behçet’s Syndrome

Currently, there are no definitive diagnostic tests available for BS, with the diagnosis dependant on the clinical criteria. The inflammatory markers commonly used in clinical practice, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), are uninformative. They may only be modestly elevated, but often there is a discrepancy between the severity of a patient’s manifestations, such as recurrent oral ulceration, and the level of CRP or ESR.

Much of the assessment of BS is designed to rule out other diagnoses. There are multiple crossovers, such as recurrent oral ulcers in SLE, inflammatory bowel disease and coeliac disease; or arthritis, which can also occur in sarcoidosis and HLA-B27 related disease. Therefore, careful clinical assessment is imperative in making the diagnosis and ruling out potential mimics; and consideration should be given to pathergy testing.

1.3.5.1 Pathergy

Pathergy is a term to describe skin hyper-reactivity to minimal trauma. In the clinic, it is induced by a minor needlestick injury, with a result considered positive when a pustule or an area of erythema develops 24 – 48 hours later at the site of injury. It was thought to be pathognomonic of BS, because of its absence in control SLE patients with mucosal ulceration in studies of 23 Israeli patients by Katzenellenbogen in the 1960s [390]. However, pathergy has subsequently been demonstrated in a number of conditions characterised by ulceration, such as pyoderma gangrenosum in which pathergy featured in a third of cases in one series [391]. Pathergy is much more common in endemic than non-endemic regions, where it can help differentiate between BS and other syndromes [392] [393] [394] [395].

1.3.6 Manifestations and treatment of Behçet's Syndrome

With the evolution of diagnostic criteria, the manifestations attributable to BS have increased in number. Mucocutaneous ulceration is still considered the hallmark of the syndrome, although attempts at new diagnostic criteria have established a subtype of BS without oral ulceration [388]. Other systemic manifestations include

the neurological, ocular, gastrointestinal and vascular systems, all of which vary significantly by gender and geographical distribution. The main manifestations and their treatments are described below, with the exception of thrombotic complications will be dealt with in section 1.6.

In view of the multi-systemic nature of BS, the management benefits from input from multiple interested specialties, such as rheumatology, ophthalmology, oral medicine and neurology. In non-endemic areas, management often requires the input of a tertiary centre with a special interest, although BS can still be managed in secondary care. The treatment of BS has been based on the underlying pathology being immune-mediated with the intensity of immunomodulatory therapy given related to clinical presentation and organ involvement.

Setting of guidelines on the management of BS is hampered by the paucity of large randomised control trials available. Consequently, treatment regimens have been dictated by expert opinion, small case series and open-labelled trials. Thus, the EULAR guidelines were based on these in 2008, whilst the Cochrane review in 2000 only accessed 10 trials that fitted their criteria for review [396, 397]. As a result, much of the evidence to support the use of therapies, particularly traditional DMARDs such as azathioprine or methotrexate, are based on small randomised control trials (RCTs) or open label studies although, there have been recent larger studies looking at the efficacy of Apremilast and Interferon-α in BS [398, 399]. As with many inflammatory conditions, psychological stress is a considerable contributor to flairs in disease, so lifestyle modification ought to be considered in all patients [400].

1.3.6.1 Mucocutaneous ulceration

Recurrent oral ulceration is the most common presenting symptom and can precede the diagnosis of BS by months or years. Ulcers often appear as crops of four or five in the buccal mucosa, and are usually less than 10mm in size, but can be larger, or herpetiform-like in nature. Ulcers are often surrounded by a ring of erythema, but this is not a diagnostically useful sign. It is unusual for ulcers to appear around the lips, so alternative diagnoses such as Stevens-Johnson syndrome should be considered when these are present. Most commonly ulcers resolve within 10 – 14 days, but can appear persistent, as they may be repeatedly replaced by new crops. If ulcers persist, then a biopsy should be considered.

Although genital ulcers do not occur in all patients, they still are a common feature [366, 389, 401]. They tend to be more common in females than males, situated mainly on the vulva and vagina, and occasionally on the cervix. In males, ulcers are usually scrotal, so alternative diagnosis such HLA-B27+ seronegative arthropathy should be considered if penile ulcers are present. Deep genital ulcers can heal with scarring, whilst oral ulcers tend not to. Other genitourinary manifestations are rare.

Maintenance of good oral hygiene is the mainstay of management of recurrent oral ulceration. This is done by use of chlorhexidine mouthwashes, which reduces bacterial growth and recurrent infections that can increase the frequency of ulcers. Topical steroids are commonly given, but there are different modes of delivery. Inhaled corticosteroids, such as beclomethasone inhalers have been used, as has triamcinolone acetonide and hydrocortisone lozenges. Often it is patient preference and experience that decides which option is chosen, although inhaled corticosteroids are more widely available and used. An RCT of 40mg intramuscular methylprednisolone given 3 weekly for 27 weeks, did not show a reduced frequency

of oral ulcers in those with active disease, and is not commonly done in practise [402].

The efficacy of colchicine was assessed in two RCTs, with mixed results [403] [404]. Overall, long-term colchicine use has not been shown to reduce the intensity of immunomodulatory therapy [405]. Despite this colchicine is often prescribed at either 500μg twice or thrice daily and patients report benefit from this therapy. It is well tolerated and can be given long term. Azathioprine is the next line in treating mucocutaneous ulceration, in those who are refractory to the combination of good dental hygiene, topical corticosteroids and colchicine. In a randomised placebo-controlled trial, azathioprine was shown to reduce the frequency of oral ulceration [406], but requires close monitoring and testing for thiopurine-S-methyltransferase (TPMT), an enzyme that metabolises azathioprine. Those with low TPMT activity may still have azathioprine, but at a lower dose. Thalidomide, cyclosporine A and methotrexate have also demonstrated effect, but with significant side effects, including teratogenicity, hypertension and glomerulopathy [407, 408] [409].

More recently, treatment of those with azathioprine-refractory disease has involved targeted agents such as etanercept, apremilast and interferon- α2a. The latter two have demonstrated a significant benefit in reducing oral ulceration [398, 410].

Overall, most patients can be controlled with simple measures, with or without colchicine. Escalation to azathioprine is not uncommon, but does not occur unless there has been a decent trial of combination good oral hygiene, topical therapy and colchicine. It is much less common to escalate further, since many respond to azathioprine. Occasionally, oral ulceration can be so severe that it warrants escalation, although it is important to reassess and consider alternative diagnoses if that is the case. Genital ulceration can be severe and is often painful, impacting

significantly on quality of life. It is not uncommon that this is the trigger to increase therapy

1.3.6.2 Skin lesions

Pustulosis, folliculitis and erythema nodosum (EN) are well-recognised dermatological manifestations of BS [411]. Pustulosis tends to be more common and clinically resembles acne. Pustules in BS share a similar microbiological flora with acne, so are not sterile [412]. EN presents typically in the lower limb, as in other conditions such as sarcoidosis and in those on the combined oral contraceptive pill, although they can also present on the upper limbs.

The management of skin manifestations often mirrors that of mucocutaneous ulceration, with positive data on azathioprine, anti-TNF, IFN-α and thalidomide. Colchicine has not been shown to be effective in treating these manifestations, whilst thalidomide is avoided since it can cause EN. In clinical practice, corticosteroids are often used, particularly in those with EN. Although intralesional corticosteroids are a recognised treatment for EN, they are not commonly used, so BS patients tend to receive them orally. Azathioprine remains the steroid sparing medication of choice.

1.3.6.3 Ocular manifestations

Ocular inflammation tends to be either posterior or panuveitis, with anterior involvement being less common and severe. Thrombosis of the branch and central retinal veins also occurs, but often coexists with retinal vasculitis [413]. Patients can present with uveitis up to two years prior to the diagnosis of BS, and it is the presenting symptom in 10 – 15% of patients. Flares of ocular inflammation are

unpredictable and can subside spontaneously, but recurrent attacks can lead to ischaemic maculopathy. This is the most common cause of permanent visual loss in BS, affecting up to 13% of patients at presentation in one series [414]. A classical feature of BS is hypopyon, which is the presence of a white fluid collection consisting of inflammatory cells, in the anterior chamber of the eye. Although dramatic in appearance, it is rarely sight threatening.

The management of BS eye disease should be led by a dedicated ophthalmologist, particularly those with posterior or panuveitis, which tend to be more aggressive. Anterior disease maybe managed with topical eye drops. The first-line remains high dose corticosteroids despite little trial evidence, with azathioprine being the corticosteroid-sparing agent of choice. Mycophenolate and ciclosporine A are proven second line agents if azathioprine is ineffective, with a preference towards mycophenolate since it is better tolerated [415-417]. Those who are unresponsive to conventional immunomodulatory therapy should be considered for biological therapy, where the majority of the evidence comes from open-labelled studies, but a recent multicentre study showed significant benefit with infliximab and adalimumab in refractory uveitis [418]. IFN-α has also been used in those refractory to corticosteroids in non-controlled studies, with some benefit [419].

1.3.6.4 Neurological manifestations

Headache remains the most common neurological symptom, occurring in up to 80% of patients [420]. Most commonly it is migrainous in type, but rarely involves aura, although tension-type headache is also well recognised in BS [421, 422]. Routine scanning of all BS patients with headache yields little. Patients should be investigated further if there is a change in quality or severity of headache, and particularly if there are other neurological signs. This is because parenchymal

disease and dural sinus thrombosis are well- recognised components of neurological BS [420, 423].

Parenchymal disease tends to be more common than neurovascular involvement, with the brainstem and mid-brain most affected. The most frequent lesion seen on MRI is a unilateral lesion extending from the brainstem to the thalamus and basal ganglia [424]. Often patients present with vestibular symptoms related to a mild version of parenchymal disease.

Neurovascular involvement is less common, affecting 20% of those with neuro-BS [420]. This includes dural sinus thrombosis, intra- and extracranial aneurysm and dissection. Typical symptoms and signs of intracranial hypertension (headache, confusional state, decreased level of consciousness and papilloedema) may be present, and should lead to urgent investigations.

It is reasonable to trial therapy instead in the form of pizotifen or β-blockade in the first instance for those with headache. For those with significant parenchymal disease, high dose corticosteroids should be instituted, with early use of DMARD therapy, which could be azathioprine or methotrexate [425]. For severe and aggressive disease, the early use of anti-TNF agents may be indicated, with infliximab the best choice since it has a rapid onset of action [420].

1.3.6.5 Musculoskeletal manifestations

Arthritis is a common feature of BS, and becomes more common as the condition progresses [361, 389, 426-429]. It tends to be palindromic, flitting from joint to joint, but is not erosive. If erosions are found then an alternative diagnosis, such as an HLA-B27+ve spondyloarthropathy should be considered. Genome-wide analysis studies (GWAS) in BS have demonstrated alterations in the ERAP1 and IL12/23

loci, both of which are associated with spondyloarthopathies, and may explain why BS tends to be associated with arthritis [430]. However, sacroiliitis is not generally a recognised feature of BS, apart from in one study [358, 386].

Patients can often be managed with simple analgesia and anti-inflammatories, rather than corticosteroids or immunomodulatory therapy [366, 368, 427, 431].

RCTs have shown benefit from colchicine over 2 years, which suppresses further flairs [379, 405]. Often this suffices and there is no requirement to increase therapy, especially since it is non-erosive. Azathioprine at 2.5mg/kg has been shown to reduce the development of arthritis compared to the placebo group at 2 years [406]. In a small 4 week placebo-controlled trial of etanercept there was trend to less arthritis in the treatment arm [410], but as with immunomodulatory therapy it is rarely required.

There have been no trials on the efficacy of methotrexate in BS. This would tend to be the first-line therapy in erosive arthropathies, and in other conditions, such as small vessel vasculitis with a significant arthritic component. In BS the necessity of using such aggressive therapy is less, so the focus should be on symptom relief especially in the context of non-erosive disease.

Widespread, non-specific pain commonly coexists with fatigue in BS and other inflammatory conditions and impacts greatly on quality of life. In one cohort, 20% of patients fulfilled a diagnosis of widespread non-specific pain syndrome, although there are substantially more who complain of fatigue in BS [432]. Often these symptoms tend to be related back to BS, but they are usually multifactorial in cause, with patients occasionally requiring input from physiotherapy, occupational therapists and clinical psychologists to alleviate their discomfort [433]. Importantly, these symptoms do not tend to be associated with other manifestations of BS.

Importantly, SAPHO (Synovitis, Acne, Pustulosis, Hyperostosis, and Osteitis) and MAGIC (Mouth And Genital ulcers with Inflamed Cartilage) syndromes crossover with BS, and should be considered if patients present with osteitis and chondritis respectively [424, 434].

1.3.6.6 Gastrointestinal manifestations

The most common symptoms patients complain of are similar to those of irritable bowel syndrome, but presentations can be variable with some having severe gastrointestinal ulceration. Therefore, in those with recurrent symptoms, wireless capsule endoscopy is helpful in visualising the whole bowel and assisting with diagnosis [435]. Occasionally, it can be difficult to distinguish BS from Crohn's, particularly when there is significant disease outside the gut, although Crohn's can be identified by the presence of granulomas on colonoscopic biopsy [436].

There are no RCTs on therapy for gastrointestinal disease in BS. A possible reason is the wide variety of presentations that can occur, from intermittent abdominal pain and occasional loose motions to severe gastrointestinal ulceration. A position paper on the use of anti-TNF in this group of patients found that all 35 went into partial or complete remission [437]. Despite this, the majority of patients require symptomatic relief only, whilst those with more severe disease can be managed as for inflammatory bowel syndrome with corticosteroids, azathioprine, mesalazine or anti-TNF.

1.3.6.7 Vascular manifestations

These include both venous manifestations (discussed in detail in Section 1.6) and arterial involvement, of which the latter tends to be aneurysmal, rather than

thrombotic. The most frequently affected site is the pulmonary artery, where aneurysms are far more common than arterial thrombosis. Despite this, the presence of aneurysm shows variability across the 'Old Silk Route' [361, 363, 438-443]. Accelerated atherosclerosis is not a feature of BS, making it unusual amongst inflammatory conditions. Cardiac involvement occurs in < 10% of patients [444].

Management of large vessel disease is based upon case reports and opinion. There are no RCTs on therapy for aneurysms and vessel stenosis. In those with pseudoaneurysms, aggressive immunosuppression is required and can be achieved either by high dose corticosteroids, cyclophosphamide or anti-TNF therapy. Most importantly is recognition of underlying inflammation as the cause for the manifestation and the need for immunosuppression. Therefore, it is important patients are appropriate immunosuppressed prior to any intervention. This is particularly so when stenting stenotic lesions, since lesions are likely to recur without it [445]. The management of thrombosis is discussed separately in Section 1.6.6.

1.3.7 Gender and age differences

BS tends to affect the genders equally, although there are differences in presentation with age and gender [362]. Male gender is associated with a greater risk of vascular involvement, thrombosis and thrombophlebitis. It has also been found to confer a higher risk of ocular involvement, cutaneous manifestations, folliculitis, papulopustular lesions. HLA-B5 carriage and a positive skin pathergy test have also been positively associated with male gender. In contrast, female gender is associated more with genital ulcers and joint involvement [429]. Further details of the studies showing differences in manifestations by gender are shown in Table 1-3.

Age at diagnosis is also an important factor governing different manifestations of BS. The frequency of ocular involvement and genital ulceration tends to be higher in those < 40 years compared with those whose age at disease onset was > 40 years [389]. Higher rates of thrombophlebitis have been observed in those <40 years [389]. In addition, clinical manifestations of BS tend to be more severe in those who developed BS when younger. When utilising a validated disease activity score in BS, it was found that disease activity, leukocyte counts, CRP levels, the cumulative probabilities of operation, admission, and corticosteroid use were significantly higher in those with onset <40 years of age than those who are older when first diagnosed.

Table 1-4 Disease manifestation by gender

Study	Vascular	Thrombus	Ocular Disease	Genital Ulcers	Papulo-Pustular	Thrombo-phlebitis	Arthralgia	Follicultis	CNS	GI disease
Alkaabi et al 2011 [446]	M>F*			M>F*						
Alpsoy et al 2007 [389]			M>F*		M>F*	M>F*				
Ames et al 2001 [447]		M>F*	M>F*			M>F*	F>M*	M>F*		
Ates et al 2005 [448]		M>F*								
Bang et al 1997 [440]				F>M						
Bonitsis et al 2015 [429]	M>F*	M>F*	M>F*	F>M*	M>F*		F>M*	M>F		
Davatchi et al 2012 [449]	M>F*	M>F*	M>F*	F>M*		M>F*	F>M*	M>F*	F>M*	M>F*
Houman et al 2001 [450]		M>F*								
Jung et al 2012 [451]				F>M*						F>M

Kural-Seyahi et al 2003 [452]	M>F*	M>F*	M>F*		M>F*		M>F*		M>F	
Salvarani et al 2007 [431]		M>F	M>F*	F>M			F>M		F>M	
Sarica-Kucukoglu et al 2006 [453]	M>F*									
Sula et al 2014 [401]	M>F		M>F	M=F	M=F		F>M		M>F	
Tugal-Tutkan et al 2004 [413]			M>F							
Tursen et al 2003 [428]	M>F		M>F	F>M	M>F	M>F	M=F		M>F	
Vaiopoulos et al 2010 [454]				M>F				M>F*		
Wu et al 2014 [455]		M>F*								
Yazici et al 1984 [392]	M>F		M>F				M>F	M>F		

GI – gastrointestinal involvement; CNS – Central nervous system involvement; M – male; F – female, * denotes a significant difference

1.3.8 Genetics

The distribution of BS along the Old Silk Route has led to the suggestion that both the prevalence, and the differing manifestations found at different parts of the route may in part be due to an underlying genetic basis. Despite this, the degree of inheritability of BS remains contradictory. While inheritability has been demonstrated in a number of familial series, a clear pattern of inheritance has not emerged [456-459]. Since males and females are equally affected, this makes sex linked inheritance unlikely. Moreover, autosomal dominant inheritance would show a stronger familial pattern, unless there was greatly reduced penetrance. Therefore, an autosomal recessive pattern of inheritance is the most likely culprit, however, a study of British and Turkish families did not reveal this mode of inheritance [457].

A further complication when studying the genetics of BS across different populations is the heterogeneity of presentation. Apart from oral ulceration, patients can present with varying collections of symptoms and signs, which varies with gender, as described, and also with geography [460]. The particular genetic 'hits' responsible for conferring these different presentations remains unknown. This would suggest that rather than being a monogenic disease, BS is a syndrome with polygenic influences, in which patients develop manifestations associated with a clutch of genetic hits.

Several loci have now been associated with BS, through linkage studies, and, more recently, genome-wide association studies (GWAS). These loci may partly explain both the distribution of disease along the 'Old Silk Route', and the regional variation in manifestations. The major loci associated are HLA B5/B51, MICA, ERAP and STAT4.

1.3.8.1 HLA B5/B51

The majority of the work for a genetic association in BS has focussed on human leukocyte antigens (HLA), found on the major histocompatibility complex (MHC) located on the short arm of chromosome 6p21.3. This complex is composed of three regions designated class I (1 Mb), class II (1 Mb) and class III (2 Mb), from centromere to telomere [461]. The first description of an association between HLA and BS was in the early 1970's, and this remains the primary genetic association with BS [374, 462, 463]. Moreover, in the early 1980s a link between *HLA-B*51* and BS was made, and, following further exploration, a link was made between Turkish BS patients, *HLA-B*51* and the spread of BS along the 'Old Silk Route' [464]. The link with *HLA-B*51* still provides the strongest evidence for genetic susceptibility in BS [465].

In itself, *HLA-B*51* is not diagnostic of BS, but remains a risk factor for BS development. The rates of HLA-B*51 carriage along the 'Old Silk Route' appear to vary, but remain higher in BS patients than controls. These rates vary from approximately 2/3 of BS patients of Ashkenazi and non-Ashkenazi Jewish descent, and Arabian extraction, compared to 9% of healthy controls (HC) [466]. [467-471]. The highest rates of HLA-B51 positivity are reported in those with mucocutaneous disease; however, as this is the most common manifestation of BS, this may be co-incidental. HLA-B51 positivity is also correlated with an increased risk for males, and a younger age of onset in both genders, but not severity of disease [472]. This has been found consistently in studies of patients along the 'Old Silk Route'.

1.3.8.2 MICA

A major member of MHC class genes termed MICA (MHC class I chain-related genes) has been shown to be associated with *HLA-B51* and may be in linkage

disequilibrium with it. *MHC class I* genes encode for cell surface glycoproteins of about 44 kDA in size that are non-covalently associated with β_2-microglobulin and present processed peptide via αβ receptors to cytotoxic T cells [473, 474]. Within the class I region, mapping studies have identified seven *MIC* loci *(MICA–MICG)*, of which only *MICA* and *MICB* are functional genes, and these are located between the *HLA-B* and *TNF* genes [475]. These encode glycoproteins that are present on intestinal epithelium, keratinocytes, endothelium and monocytes, and they are recognised by γδT cells at the intestinal epithelium [476, 477]. This association is important, since studies have shown that γδT cell responses were preferentially elicited in 76% of BS patients compared with 2.6% of controls (consisting of healthy volunteers, those with recurrent oral ulceration and those with other systemic diseases), demonstrating a role for MIC glycoprotein [478]. *MICA* has been associated with BS and *HLA-B*51*, in many different BS patient populations, including European Caucasians, Koreans, Jordanians, Israeli, Greek, Japanese and Koreans [466, 479-489]

Overall, MICA, particularly the A6 allele, has been shown to be independently associated with BS, and is strongly associated with HLA-B51. It is unclear whether the interaction between MICA-A6 and HLA-B51 led to specific clinical manifestation of BS or increased severity. However, since it is specifically expressed in the intestinal epithelium, keratinocytes, endothelium and monocytes, it suggests that its effects in BS are widespread. Therefore, it may have a role in mucocutaneous ulceration and venous inflammation, which are characteristic of BS.

1.3.8.3 ERAP

BS shares some similar clinical features with seronegative spondyloarthropathies, namely ankylosing spondylitis, psoriatic arthritis and Whipple's disease [490]. All

demonstrate seronegativity for rheumatoid factor, lack of nodules, polyarthritis and the presence of sacroiliitis. Sacroiliitis has been reported, but remains an uncommon feature of BS and has not been part of any diagnostic criteria [358, 376, 385, 388].

Further evidence for BS as a spondyloarthropy comes from the association with the *ERAP* gene. ERAP (*endoplasmic reticulum aminopeptidase*) is an enzyme involved in the final trimming of the terminal ends of peptides in the endoplasmic reticulum for presentation of MHC class I molecules and [491]. It has been shown to have a role in ankylosing spondylitis [492].

Two SNPs in *ERAP1*, encoding *ERAP1* p.Asp575Asn and p.Arg725Gln alterations, recessively conferred disease risk for BS. These findings were replicated in 1,468 independent Turkish and/or 1,352 Japanese BS samples (combined meta-analysis $p < 2 \times 10^{-9}$) [371]. The authors found that there was a recessive association between the *N575* and *Q725* variants of *ERAP1* and BS, and it was the *Hap10 ERAP1* allotype that conferred risk for BS. Of note, those with the *N575/Q725/Hap10* have a decreased susceptibility for psoriasis and ankylosing spondylitis, whilst having an increased risk of BS [493].

Importantly, there is a significant association between *HLA-B*51* and *ERAP1* ($p = 9 \times 10^{-4}$), and it is possible that this interaction, like that between ERAP1 and HLA-B27 in ankylosing spondylitis, may lead to a variation of antigen presentation by MHC Class I molecules and, therefore, a variation in BS presentation [371, 372, 493, 494].

1.3.8.4 IL-12/23

Studies of the cytokine profile in BS has shown a variation between T_h1 and T_h2 responses, with important roles also for T_h17 responses and T_h0 cytokines. T_h1 responses are mainly for cell-mediated immunity, fighting bacterial infections and cancer, whilst T_h2 role is mainly in humoral immunity and fighting parasitic infections [495]. Imbalance between T_h1 and T_h2 responses can lead to different types of disease, with predominant T_h1 responses leading to inflammatory conditions such as autoimmune hepatitis, inflammatory bowel disease or pyoderma gangrenosum, whilst predominant T_h2 response can lead to allergic conditions such as atopy, drug allergy and bronchial asthma. T_h1 cytokine profile is predominant in active BS, consistent with the significant role that IL-12 plays in this condition compared with IL-4. IL-12 and IL-4 are potent triggers of T_h1 and T_h2 responses respectively.

CD3+ T cells with intracellular synthesis of IFN-γ have been shown to be increased on flow cytometric analysis in active BS compared with quiescent disease and healthy controls, whilst serum IL-12 was also increased in active BS and correlated well with disease activity [496]. The imbalance in T_h1 and T_h2 cytokine profiles seem to be more prevalent in active BS than in healthy controls [497] [498] [499].

It has therefore been demonstrated both at the transcription and protein level that the T_h1 signature predominates over the T_h2 signature, which is consistent with the finding that IFN-γ plays a significant role in the manifestations of BS. Since IL-23 is a dimer between the IL-12p40 and IL-12p19 subunits, there is a strong link between IL-23 and IL-12 cytokines. This has been shown in the various BS GWAS to be the case and confirmation of 6 SNPs at the IL-23 receptor–IL-12 receptor β2 (IL23R–IL12RB2) locus, being preferentially associated with the development of BS in Iranian patients compared with healthy controls [500]. Increased IL-23 mRNA has been shown to be increased (6.7-fold) in BS patients with erythema nodosum

compared with healthy controls, and the same study found an equivalent increase in psoriatic patients (8.5-fold), but there was no concomitant increase in the serum level of either cytokine [501].

1.3.8.5 STAT4

Signal transducers and activators of transcription (STATs) are transcription factors that provide a direct link between the cytokine receptors and cytokine induced gene transcription [502].

Stat4 is located on chromosome 1, alongside *Stat1*, which is involved in responses to Type I and II interferons [503, 504]. Human T cells constitutively express low levels of STAT4 protein by immunoblotting, but this dramatically increases with phytohaemagglutinin and also with IL-12 stimulation [505]. In *Stat4* deficient mice there is defective T_h1 development, consistent with a significant role for the interaction between IL-12 and STAT4 [504].

It is this interaction that explains the increased *Stat4* signal in BS patients, in GWAS and other genetic studies. In Han Chinese BS patients, GWAS and replication studies identified a susceptibility locus around STAT4 (single nucleotide polymorphisms rs7574070, rs7572482, and rs897200; $p = 3.36 \times 10^{-7}$ to 6.20×10^{-9}). The latter of these polymorphisms results in regulation of IL-17, which is notable since IL-17 has been implicated in BS and is a possible therapeutic target [506]. A phase III randomised controlled study of Secukinumab (mAb to IL-17), in Behçet's uveitis showed no significant difference in recurrence rate between treatment and placebo arms. This is possibly due to the BS patients being treated with corticosteroids or concomitant immunomodulatory therapy [507]. There are also

gene–gene interactions between SNPs in IL23R, IL17A, and STAT4, with an association between STAT4 and ileocaecal valve involvement in BS [508].

1.3.8.6 Genome Wide Association Studies

Genome Wide Association Studies (GWAS) has been developed to identify common (minor allele frequency ≥ 1%) single nucleotide polymorphisms (SNP) associated with specific disease phenotypes by using low-cost, high throughput genotyping platforms. There have been multiple genome wide association studies performed in BS, including Turkish, Iranian, Saudi, Japanese and Korean patients [371, 374, 430, 462, 506, 509-513].

The GWAS in BS have identified several SNP, each associated with a small increase in risk, and determining further genetic associations with BS. This gives an insight into the potential aetiology of manifestations and how particular SNP interact with *HLA-B*51* and *MICA-A6*, for example, in causing the phenotypic differences that occur in BS patients across the world. It is also possible that SNP may in future allow for a calculation of risk of further BS complications allowing guidance of therapy, whether that being increasing or decreasing immunomodulatory therapy, depending on the collection of SNPs. Additionally, these findings may lead to new therapeutic targets worthy of future research.

1.4 Thrombosis in Behçet's Syndrome

1.4.1 Epidemiology

The original triple symptom complex as described by Dr. Hulusi Behçet and early case reports did not describe thrombosis as part of BS [357, 384]. However, it has come to be a well-recognised feature that causes considerable morbidity and is associated with increased mortality. Mason and Barnes described 6 patients (24%) with thrombosis, whilst Lehner described 5/21 (19%) [358, 514]. Both of these studies were based on BS patient cohorts in London and neither described vena caval lesions, but when they occur they are often obstructive [515]. Studies quote that venous thrombosis occurs in 7 – 37% of patients, with an average of 20%, with diagnostic criteria appearing to be important in variability of these numbers [439, 516].

Turkish studies have demonstrated an increased prevalence of venous thrombosis in BS; particularly in younger males, those with eye disease and positive pathergy testing, a phenotype susceptible to more aggressive disease [438, 439, 452]. Rates of thrombosis have ranged between 9.9%-23.9% [517, 518].

Thrombosis is more common in males than females, with a two-decade outcome study of 387 Turkish BS patients showing that 15.7% of patients had a thrombotic event, 22.8% male and 1.8% female, with the majority (95%) being venous [452]. In this same cohort, 42 patients died, 39 of who were male, 40.5% of who had vascular involvement compared to 18.8% of those alive [452]. This has been shown in a number of other studies, with males on average being 9 times more likely to develop thromboses than females [446, 450]. This may be reflective of more aggressive disease in males, as evidenced by their higher rates of inflammatory eye disease, another marker of more aggressive disease [361, 366].

Rates of thrombosis in other countries vary widely, with at least come of this variability likely due to changes in diagnostic criteria. An Iranian study of 6500 BS patients showed that 5.57% develop superficial thrombophlebitis, 1.1% deep vein thrombosis and only 0.154% develop arterial thromboses [366]. Such a reduced rate of thrombosis is probably due to utilising the ICBD criteria for the diagnosis of BS, potentially diluting the population [388, 519, 520]. In an Omani study of 40 patients, 9 had vascular lesions, of which only 2 were venous lesions, with more having arterial lesions, although this is an unusual finding [446].

In a study of 766 BS patients in Beijing, 93 (12.1%) had thromboses, of which 78 (10.1%) developed deep vein thrombosis [455]. As with other groups, there was a significant male preponderance (6:1 in favour of males), but there were only 4 patients with superficial thrombophlebitis. This is likely not to be a true reflection, since there are more patients with superficial thrombophlebitis than deep vein thrombosis [439, 453, 521]. Rates of thrombosis were higher in a Tunisian study (38.9%), but showed the same male preponderance and association with pathergy [450, 455]

1.4.2 Thrombosis in Behçet's Syndrome and thrombophilia

The underlying cause of thrombosis in BS is unclear. It is unusual amongst chronic inflammatory conditions in that it affects vessels of any size and causes thromboses in both venous and arterial systems, albeit more commonly in veins [348, 362, 522-524]. Thromboses occur anywhere in BS with large vessel involvement, cerebral vein thrombosis, Budd-Chiari syndrome and mesenteric vein thrombosis all reported [455, 518, 525-527].

Assessing for an association with hereditary thrombophilia has not elucidated a common connection. Several studies have assessed a variety of thrombophilias, and have not found any consistent evidence of a clear role in those with thrombosis. Further details of these studies, and the factors they assessed are shown in Table 1-5.

Only one study specifically addressed the role of thrombophilias in those with recurrent venous thrombosis, performing thrombophilia analysis of 18/24 patients with recurrent venous thrombosis, from a cohort of 402 BS patients. They found that all but 3 were male with a mean age of 33.3 years, and an average disease duration of 5.3 years [517]. A third of patients were protein C deficient, a third were protein S deficient, 5/11 had Factor V Leiden mutation, 2 patients had Activated Protein C resistance, 2 had prothrombin gene mutation and 2 had either hyperhomocysteinaemia or methylene tetrahydrofolate reductase deficiency [517]. They concluded that thrombophilias may play a role in BS patients with recurrent venous thromboses, but not in those with a single episode.

1.4.2.1 Hyperhomocysteinaemia

Homocysteine levels have been extensively studied, with opinion remaining divided. Smaller studies have tended to show a positive correlation between plasma homocysteine, and vascular and thrombotic complications in BS, but this has not been replicated in larger studies. This is exemplified by studies such as a Turkish study of 45 patients and 40 healthy controls. This showed that thromboses were more common in males than females (46.7% v 13.3%, $p < 0.05$), whilst serum homocysteine levels were significantly higher in thrombotic BS compared to non-thrombotic BS and healthy controls (13.8 ± 5.7 v 9.9 ± 2.6 v 9.1 ± 2.5, $p < 0.001$ and <0.0001 [448]. In another Turkish study there was no significant difference in

plasma homocysteine levels between BS patients with and without a history of thrombosis ($p = 0.86$) [528]. It has also been shown in the Women's Health Initiative study of 5522 patients with cardiovascular disease or diabetes that successful reduction of homocysteine with a combination of folic acid, vitamins B_6 and B_{12} versus placebo did not alter the rates of death from cardiovascular events (519/2758 v 547/2764, respectively) nor VTE (40/2758 v 37/2764, respectively). This suggests that lowering homocysteine levels does not reduce the risk of vascular problems [529].

Table 1-5 Thrombophilia prevalence and thrombosis

Study	Population	Patients	FVL	HHC	APC	Prot C	Prot S	PT	AT	MHTFR	Other
Aksu et al 2001 [530]	Turkey	84 BS 36 HC 29 RA on MTX		+							64% of BS with thrombosis had HHC
Ates et al 2005 [448]	Turkey	45 BS 40 HC		+							
Ates et al 2003 [531]	Turkey	71 BS (27 Th^+) 91 HC	+/-					-			Assessed 3 specific mutations
Canataroglu et al 2003 [532]	Turkey	90 BS (14 Th^+) 60 HC		+							
Caramaschi et al 2010 [533]	Italy	30 BS (9 Th^+)	-					-			6 with recurrent thromboses had a thrombophilia
Dagan et al 2012 [534]	Israel	54 BS	-					-		-	
Epinosa et al 2002 [443]	Spain	38 BS (13 Th^+) 38 VTE 100 HC	-			-	-	-	-		

Feki et al 2004 [535]	Tunisia	59 BS 118 HC		-							
Gonul et al 2009 [536]	Turkey	45 BS 69 HC		-							Most pt had mucocutaneous, not vascular disease
Gul et al 1999 [537]	Turkey	64 BS (32 Th^+)	+					+			
Gul et al 1996 [538]	Turkey	64 BS 107 HC	+								
Gurgey et al 1998	Turkey	34 BS 79 HC	-								
Hodeib et al 2010 [539]	India	25 BC 15 HC		+							
Korkmaz et al 2002 [528]	Turkey	74 BS 35 HC 14 RA on MTX		+							No difference in levels between Th^- and Th^+
Leiba et al 2004 [540]	Israel	107 BS	-	-				-		-	

Study	Population	Patients	FVL	HHC	APC	Prot C	Prot S	PT	AT	MHTFR	Other
Lesprit et al 1995 [541]	France	15 BS			-						
Navarro et al 2004 [542]	Spain	39 BS 78 HC			+		+		+		
Ricart et al 2006 [543]	Spain	79 BS (23 Th^+) 84 HC	-					+			
Silingardi et al 2004 [544]	Italy	118 BS 132 HC	-					-			
Tursen et al 2001 [545]	Turkey	21 BS 95 HC	-					+			
Verity et al 1999 [546]	Jordan/Israel	106 BS 120 HC	+								

KEY: FVL – Factor V Leiden; HHC – Hyperhomocysteinaemia; APC – Activated Protein C; Prot C – Protein C; Prot S – Protein S; PT – Prothrombin; AT – Antithrombin III; BS – Behçets Syndrome; HC – healthy controls; RA – Rheumatoid arthritis; MTX – Methotrexate; Th^+ - history of thrombosis; Th^- - no history of thrombosis; **+** - association found; **-** no association found

1.4.3 Behçet's Syndrome and endothelial dysfunction

Inflammation leads to endothelial dysfunction and oxidative stress in BS. Superficial thrombophlebitis in BS is characterised by clinical features of inflammation surrounding the thrombus. Thrombi in BS tend to adhere strongly to inflamed endothelium and have a long tail. This adherence may explain why the reported risk of pulmonary emboli is lower than in other patients with venous thrombi [446, 547]. Markers of oxidative stress, including high sensitivity CRP and gamma-glutamyltransferase, are raised in BS and are related to disease activity [548]. Oxidative stress has been found to be associated with endothelial dysfunction in a study measuring brachial artery flow mediated dilation in BS and healthy controls [549]. Brachial artery flow was impaired and responded to the introduction of nitroglycerine and, in particular, vitamin C, which is known to be a scavenger of superoxide anion radicals [549].

Von Willebrand Factor is a marker of endothelial dysfunction, as is Endothelin-1 and impaired nitric oxide activity. Von Willebrand Factor is elevated in BS, but it is not associated with increased inflammation or disease activity [547]. Endothelin-1 levels are increased in BS patients and have been demonstrated to rise with disease activity [539]. Studies have also suggested that nitric oxide activity rises in BS [550]. This is counterintuitive, as impaired nitric oxide activity leads to vasoconstriction, platelet aggregation and monocyte adhesion, all of which are known to promote thrombosis. It is also known that reduced nitric oxide causes platelet aggregation in BS [549]. Low thrombomodulin is another feature of endothelial dysfunction, but there is little evidence that this is decreased in BS [443].

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and marker of endothelial dysfunction. VEGF levels are significantly higher in BS and are associated with carotid intima-media thickness [551]. Bozoglu *et al* investigated

whether VEGF and monocyte chemoattractant protein1 (MCP-1) increased the risk of thrombosis in BS. Both VEGF and MCP-1 expression was increased in BS patients with acute and chronic thrombosis compared to disease and healthy controls, but VEGF was significantly higher in those with acute thrombosis versus those with chronic thrombosis [330]. Thus, VEGF is a potent contributor to the procoagulant state in BS.

Inflammation also increases the risk of thrombosis in ways other than endothelial dysfunction that are analogous to atherosclerosis. When an atherosclerotic plaque ruptures, thrombus forms via a TF dependent pathway [552]. CRP increases TF and, in vivo, also enhances TF expression by vascular smooth cells [167, 553]. TF pathway inhibitor (TFPI) is an anticoagulant protein synthesised by the vascular endothelium that modulates the TF dependent pathway of coagulation. TFPI is known to decline with rising CRP and is reduced in BS patients compared with healthy subjects. In BS, the baseline level of 136.0 ng/ml of TFPI is similar to that of healthy subjects, at 149.5ng/ml. However, when given a heparin infusion, TFPI levels only rise 44.7% rise in BS patients, versus almost 400% rise in healthy controls [554]. Fibrinolysis is also impaired in BS, so thrombi tend to persist. The euglobulin clot lysis time is demonstrably increased in patients with raised CRP levels [543].

1.4.4 Fibrinolysis in Behçet's Syndrome

Impaired fibrinolysis in BS has been well documented and is an additional explanation to why thrombosis persists in BS. Broadly, two inhibitors of fibrinolysis have been shown to be increased in BS: plasminogen activator inhibitor-1 (PAI-1) and thrombin activatable fibrinolysis inhibitor (TAFI). PAI- 1 is a serine proteinase inhibitor, and the main physiological inhibitor of the endogenous fibrinolytic system,

and levels are associated with increased risks of thrombosis and cardiovascular events [555-557]. TAFI is a carboxypeptidase that is activated by thrombin, which cleaves lysine residues on partially degraded fibrin thereby disrupting the binding sites for plasminogen [558]. Both PAI-1 and TAFI have been shown to be increased in BS patients compared to healthy controls ($p < 0.001$ and $p = 0.022$, respectively), with TAFI significantly associated with BS patients who have had a thrombotic episode ($p = 0.024$) [559].

The impact of the rise in PAI-1, is possibly to reduce the level of tissue plasminogen activator (tPA) [560, 561], although another study showed no difference in tPA level between healthy controls, BS patient and BS patient with a history of thrombosis [443]. Even so, the persistent finding of a raised PAI-1 level would cause impaired fibrinolysis. This was assessed by rotational thromblastometry (ROTEM), which assess the kinetics of both clot formation and fibrinolysis, and found not only that PAI-1 levels were increased in BS patients, but that the time to fibrinolysis was also increased in BS *versus* healthy controls [562].

It is possible that impaired fibrinolysis is a significant reason why BS patients have more persistent thrombi than healthy controls. Notably, none of these studies were able to passively identify patients with thrombosis and the findings were in BS patients compared with healthy controls.

This test measures, amongst other proteins, plasminogen activator inhibitor – 1 (PAI-1) and PAI-1 levels are reduced in BS [543]. It is the combination of TFPI and TF imbalance and impaired fibrinolysis that confer increased thrombotic risk and persistence of thrombi.

1.4.5 Laboratory markers and thrombosis

To date, laboratory tests have not been helpful in identifying those with an increased risk of thrombosis. CRP and ESR have been assessed but have not been predictive, with many BS patients having low CRP when having a thrombotic episode. One study showed a significant increase in CRP in BS patients compared to healthy controls (2.3 *versus* 1.4 mg/L, $p < 0.001$), but the magnitude of rise was small and CRP did not associated with the thrombotic phenotype of BS [542]. Another study reported testing for CRP and ESR, but simply did not report their findings, presumably because there was no significant association found [446]. Moreover, a further study of Turkish patients did find an association, but this was unlikely to be the case since the 95% confidence intervals were so wide. However, inflammation clearly plays a role with increased endothelial dysfunction, as represented by raised von Willebrand Factor (vWF) and nitric oxide [563, 564], inflammatory superficial thrombophlebitis [524] and the strong adherence of thrombus to the inflamed endothelium [565].

As documented in Table 1-6, there have been no definitive associations between thrombophilia and thrombotic risk in BS. Moreover, standard coagulation tests in BS are of little value. There have been efforts made looking at alternative tests such as ROTEM and CAT assay that have been promising [562]. However, neither test has penetrated common clinical practice, although this may change in future.

1.4.6 Management of thrombosis in Behçet's Syndrome

Although the cause of BS remains unknown, management strategies have focussed on treating it with immunomodulatory therapy rather than with anticoagulation. A small prospective Korean study showed no difference in recurrence in thrombosis

between immunomodulation only and immunomodulation and coagulation arms, whilst those who received anticoagulation only were more likely to have recurrence of thrombosis [566]. Larger retrospective studies of French and Turkish cohorts showed similar results, with increasing frequency of recurrence in those solely on anticoagulation [567, 568]. These findings support prevalent thinking in managing thrombosis in BS, such that EULAR guidelines recommend immunomodulatory therapy over anticoagulation as well [396]. However, this recommendation was based on two small studies that evaluated azathioprine and cyclosporine A in reducing the frequency of thrombosis [415, 569]. There is a need for further study in this area.

One of the reasons put forward for not using anticoagulation is the low risk of embolisation that occurs in BS. This has been shown in a number of cohorts, with two Turkish studies reporting no episodes of pulmonary embolus, whilst one Iranian cohort showed 10 episodes of pulmonary embolus in 6500 BS cases [366, 453, 518]. The most likely reason is that deep venous thrombi are strongly adhered to the inflamed endothelium, as has been shown in post mortem examination of a BS patient [565]. These thrombi have short tails, so are much less likely to embolise compared to thrombi in other conditions.

In non-endemic regions management is made more complicated by patients being anticoagulated prior to the diagnosis, because the rarity of the condition means there can be a delay between the thrombotic episode and diagnosing BS. As such, EULAR recommendations are not applicable, since it is a more complicated decision to cease anticoagulation once it has been commenced. When patients do present to a clinician with an interest in BS, it is important that they have either magnetic resonance or CT angiography to rule out the presence of arterial pseudoaneurysms [363, 438, 439, 442, 443]. Moreover the presence of pulmonary

artery pseudoaneurysm and deep venous thrombosis (Hughes-Stovin syndrome) are well recognised and anticoagulation in these patients can cause catastrophic haemorrhage [570].

Currently, there are no tests to guide patient management and risk whilst on anticoagulation or immunomodulatory therapy. Much of the time, the patient's clinical response to therapy is the determining factor in either intensifying or downgrading therapy. Often that can be due to the presence of recurrent oral ulceration or other features of BS. There is a clear need for biomarkers or serological tests to identify those at risk of thrombosis and risk manage those on therapy.

1.5 Aims of Thesis

The aims of this thesis were:

- Establish an assay that would be able identify and quantify MP in BS patients, and those with a history of thrombosis, and compare with healthy controls and those BS patients without a history thrombosis (Chapter 3)
- Quantify total, CD14+ and CD66b+ MP, especially those that co-stain with TF, in BS patients compared with healthy controls, and in BS patients with a history of thrombosis compared to those without (Chapter 4).
- Quantify and identify CD62P+ and GSL-1 MP, and those that co-stain with TF, in BS patients compared with healthy controls, and in BS patients with a history of thrombosis compared to those without (Chapter 5).
- Evaluate tissue MP function with coagulant assays, such as coagulation lysis, thrombin generation and a proprietary MP TF function assay (Chapter 6).
- Assess for the number of TFPI+ MP, by flow cytometry and compare with total and TF+ MP (Chapter 7).

2 Methods and Materials

This section covers the general methodology used. Specific variations will be mentioned in the individual chapters.

2.1 Clinical study design

This was a cross-sectional retrospective study incorporating BS patients, with and without a history of venous thrombosis, and matched healthy controls.

2.2 Eligibility criteria

I studied BS patients aged over 18 years who fulfilled criteria for diagnosis as set by the International Study Group Criteria 1990 [385]. Patients were recruited from BS clinics at both the Imperial College Healthcare and St. Thomas' Healthcare NHS Trusts from August 2011 – November 2014. I collected data on participants as documented in Appendix 1. Data collected included basic demographic details on all participants, in addition to details of manifestations of BS, including dermatological, ocular, cardiac, gastrointestinal and neurological manifestations. BS activity was assessed using the Leeds BS Disease Activity Index, which scores activity on a 0 – 12 point scale [571]. I also recorded any history of thrombosis or cardiovascular events, along with medications, including immunomodulatory therapy and tumour necrosis factor inhibitors.

A diagnosis of thrombosis was confirmed from patient testimony and clinical records. I excluded patients with significant morbidity, which included malignancy, extensive cardiovascular disease, other autoimmune conditions and acute or chronic infections.

Healthy controls were recruited using word of mouth and from the healthy control database administered by the National Institute of Health Research/Wellcome Trust Imperial Clinical Research Facility (CRF). Healthy controls have been collected by the CRF that fulfil general criteria for healthy controls and have no comorbidities. Matching was achieved by interrogating the database. All patients collected and their details are referenced in Appendix 1.

2.3 Microparticle processing

The preparation and processing of samples for MP was performed using a well validated and published protocol from the Brogan group that we have an ongoing collaboration with, and it continues to be used [204, 344, 572, 573].

2.4 Peripheral blood collection

After gaining informed consent, 20mls of peripheral blood was collected from each healthy volunteer and patient. Peripheral blood was collected using aseptic non-touch technique as per local guidelines with a disposable tourniquet. A 21-gauge butterfly needle was used to collected peripheral blood into a 20ml slip tip syringe (BD, cat no. 302831). Blood was then gently transferred into a citrated 50ml conical tube (Corning Life Sciences, cat no. 430829). Peripheral blood samples were citrated using sodium citrate 4% w/v (Sigma Aldrich, cat no. S5770–50ml) at a concentration of 10%. From some of the healthy volunteers, 20mls of peripheral blood was collected as above and incubated with lipopolysaccharide (LPS) (Sigma, cat no. L2880-10MG) at a concentration of 10ng/ml for 4 hours at 37˚C. This led to LPS-stimulated peipheral blood.

2.5 Preparation of platelet poor plasma

Citrated peripheral blood samples underwent differential centrifugation. Initially, samples were centrifuged at room temperature at 700g for 10 mins, with deceleration set at the lowest setting of 0. This was to prevent remixing of the blood due to too rapid a deceleration and gave platelet-rich plasma (PRP), with cells sedimented at the bottom.

The PRP was decanted carefully into a clean 50ml conical tube using a 10ml pipette, so as not to contaminate it with erythrocytes and other cells. The PRP was centrifuged at 5000g for 10 minutes, with deceleration set at the maximum setting, 9. Plasma was decanted carefully into a further clean 50ml conical tube, so as not to contaminate the plasma with the sedimented cellular pellet, and centrifuged for a second, and final, time at 5000g for 10 minutes, again with deceleration set at 9. The plasma was decanted carefully again into a further clean 50ml conical tube and was platelet poor plasma (PPP). The PPP was then aliquoted into Eppendorf tubes in 500µl volumes and frozen at -80˚C ready for further study. LPS-stimulated peripheral blood samples also underwent this process

2.6 Preparation of microparticles

When required, PPP samples were thawed in a water bath set at 37˚C for 1 – 2 minutes. This was done in preference to thawing at room temperature to prevent the development of cryoprecipitates, which are plasma proteins that are created when the transition from solid to liquid phase occurs too slowly [574]. Once thawed, samples were centrifuged at 17000g for 60 minutes. Whilst the samples were being centrifuged, AnV-binding buffer was prepared by diluting 1 part 10 x AnV-binding

buffer (BD Pharmingen, cat no. 556454) with 9 parts molecular grade water (Sigma Aldrich, cat no. E7023-500ml). This was then stored on ice prior to use.

Once the hour centrifugation step was complete, the samples were carefully removed, so as not to invert them and disturb the pellet. Then, using a 200µl pipette, 480µl of supernatant was removed. This was MP poor plasma (MPPP), which was then either discarded or stored for later use, leaving 20µl at the bottom of the Eppendorf containing the MP pellet. The pellet was gently re-suspended to 500µl by the addition of 480µl of 1 X AnV-binding buffer, giving the MP suspension. The MP suspension was stored on ice and used within 2 hours for flow cytometry.

2.7 Isolating monocytes

2.7.1 Histopaque separation

50mls of peripheral blood was collected from healthy volunteers into conical tubes, as previously described. The sample was then centrifuged for 10 mins at 700g, with acceleration set at 9 and deceleration set at 0. The conical tube was then taken into a sterile negative pressure hood, in which the rest of the work was done to prevent pathogen contamination and all reagents used were sterile. Following centrifugation, the blood had separated into PRP and cells. The PRP makes up approximately one half the volume, and was gently removed using a 10ml pipette, being careful not to contaminate the PRP with the cells, leaving 1ml of PRP above the cells. The PRP was utilised to prepare PPP as per section 2.4.

The remaining blood was divided into two 50ml conical tubes, with 12.5mls in each. These two volumes of blood were re-suspended to 35mls using phosphate buffered saline (PBS) (Life Technologies Gibco®, cat no. 10010023). 15mls sterile filtered Histopaque (Sigma Aldrich, cat no.1077) was added to 50ml conical tubes. Blood

was layered onto the Histopaque, being careful not to mix the blood with Histopaque, using a 10ml pipette at very slow speed. Once sitting on the Histopaque layer, erythrocytes started to sediment almost immediately.

Once the layering was complete, the samples were centrifuged at 700g for 20 minutes with deceleration set at 0. Once complete there was a visible cellular layer in the middle of the tube, containing the mononuclear cells, with erythrocytes at the bottom of the tube. Being careful not to contaminate with erythrocytes, this cellular layer, and the surrounding clear fluid, was carefully decanted using a 5 ml pipette. Approximately, 25ml was taken from each tube, and the erythrocytes were discarded.

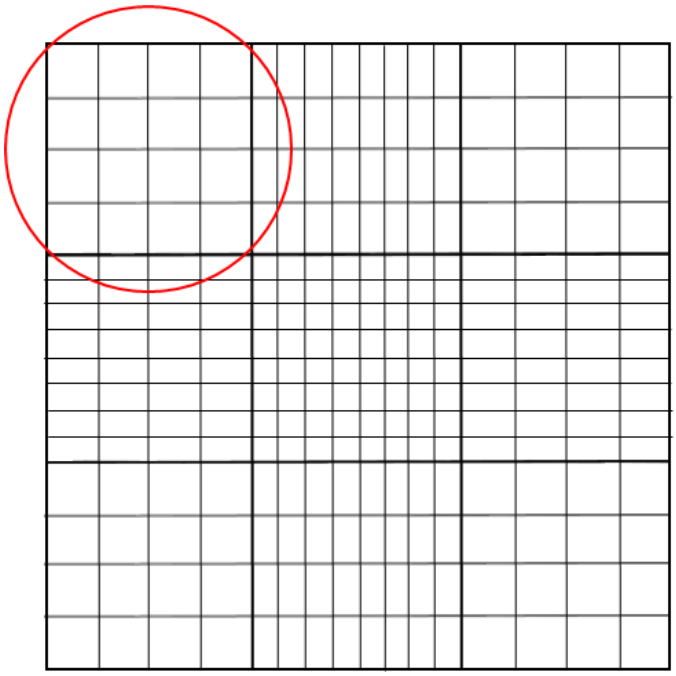
The samples then underwent sequential centrifugation and washing with PBS. These steps were 700g, 500g and 300g all for 10 minutes with deceleration set at 9. Once each centrifugation step was complete, the supernatant was decanted using a 5ml pipette, being careful not to disturb the cellular pellet, and discarded. The cellular pellet was gently re-suspended in PBS and then underwent the next centrifugation step. At the end of the first step, all the tubes from a single volunteer were combined. At the end of the last step, the supernatant was decanted in preparation for monocyte isolation.

2.7.2 CD14 MICRObeads to isolate monocytes

MACS® buffer was made using 50mls PBS, 0.5% bovine serum albumin (Sigma Aldrich, cat no. A9306) and 0.5M EDTA (Sigma Aldrich, cat no. 03690), which was stored on ice or refrigerated before use. The cellular pellet was re-suspended in 10mls MACS® buffer and the cells were counted using a haemocytometer. Using a pipette, 10µl of the suspension was drawn up and expelled over the

haemocytometer chamber. A coverslip was placed over the chamber and the haemocytometer chamber grid was visualised using the 10X objective of a microscope. The haemocytometer is designed so that the number of cells in one set of 16 corner squares is equivalent to the number of cells x 10^4 /ml (Figure 2-1).

Figure 2-1 Haemocytometer used to measure cell count during monocyte isolation



This shows the haemocytometer grid. Cells were counted in the corner 16 squares (circled) with a hand talley counter. This was repeated in the other 3 corners.

Using a hand tally counter, the number of cells was counted in a corner of 16 squares, and was repeated in the other 3 corners. The totals cells/ml was calculated thus:

$$Total\ cells = \frac{total\ counts\ for\ all\ corners\ \times\ 10^4}{4}$$

The cellular suspension was then centrifuged at 300g for 10 minutes and the supernatant was removed, being careful not to disturb the cellular pellet. The pellet was then re-suspended in MACS buffer at 50 - 80µl MACS buffer per 1 x 10^7 cells. 20µl of CD14 MICRObeads (Miltenyi Biotec, cat no. 130-050-201) were added per 1 x10^7 cells, mixed well and incubated for 15 minutes.

1 – 2mls of MACS buffer per 1 x 10^7 cells was added to the cell and bead suspension and this was centrifuged at 300g for 10 minutes, with deceleration set at 9, and the supernatant was discarded following this step. The cellular pellet was re-suspended in 500µl MACS® buffer and stored on ice, ready for magnetic bead separation of CD14+ mononuclear cells.

The MS column (Miltenyi Biotec MS positive selection column, cat no. 130-042-201) was set up by attaching it to a magnet. The MS column was primed using 1.5ml of MACS® buffer. Once primed, the 500µl cellular suspension was passed through the MS column, then the column was washed with 1.5mls MACS® buffer and was removed from the magnet. Subsequently, the column was placed carefully over a sterile 15ml conical tube, being careful not to contaminate it, and 1ml MACS® buffer was added to the column. The buffer was passed through with the plunger provided, leaving the CD14+ mononuclear cells in the 15ml Falcon tube. The cells were counted using the haemocytometer as before.

The cellular suspension was centrifuged at 300g for 10 minutes again, with deceleration set at 9, and then re-suspended in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies Gibco® +L-Glut, 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Phenol Red, cat no. 22320-030), foetal bovine serum and penicillin and streptomycin antibiotics, which had been warmed to 37˚C, making a concentration of 1 x 10^6 cells/ml.

2.8 Flow cytometry

All flow cytometry was performed Beckmann Coulter Cyan® ADP 9 colour flow cytometer (Beckman Coulter, cat no. CY20131) and the acquiring software was Summit 4.3, which is the proprietary software that came with the flow cytometer.

2.9 Preparing the flow cytometer

Some flow cytometers can resolve particles as small as 300nm in diameter, but most resolve consistently sizes of 500nm [244, 268]. With most flow cytometers, a lower limit of detection can be set, which is known as the threshold, allowing detection of cells or particles of interest. The lowest threshold that can be set with the Beckmann Coulter Cyan® ADP flow cytometer is 0.01% and the threshold set in this study was 0.02% on forward scatter. When setting such a low threshold, background noise became more apparent, so it was important that the flow cytometer was carefully cleaned prior to running samples. The sheath fluid used was filtered ddH_2O. Prior to running samples, molecular grade water and FACS clean (Beckmann Coulter, Coulter Clenz®, cat no. 8546931) was run through the system for 30 minutes each, to reduce background noise. Background was checked on the forward *versus* side scatter dot plot and, particularly, in the gated area that was below 1.1 µm in diameter. The background noise was sufficiently low when there were less than 100 events in this area when running molecular grade water only. This gave enough time for the lasers to warm up, which, according to Beckmann Coulter engineers, took 20 minutes to occur.

2.9.1 Annexin V and monoclonal antibodies

Initial set up experiments were done to confirm the concentrations of mAbs and protein to be used (see section 3 for details). The mAbs used were phycoerythrin (PE) conjugated anti-CD14 (Invitrogen, cat no. MHCD1404, clone Tük4), fluoroisothiocyanate (FITC) conjugated anti-CD142 (tissue factor, TF) (AbD Serotec, cat no. 090312, clone CLB/TF-5), PE conjugated anti-CD66b (Biolegend, cat no. 305106, clone G10F5), PE conjugated anti-CD62P (eBioscience, cat no. 12-0628-42, clone AK-4), PE conjugated anti-CD144 (eBioscience, cat no. 12-1449-82, clone 16B1) and allophycocyanin (APC) conjugated anti-CD162 (P-selectin glycoprotein ligand-1, PSGL1) (eBioscience, cat no. 17-1629-42, clone FLEG). To detect MP, AnV protein (Biolegend, cat no. 640918) was used. AnV binds to phosphatidylserine that results from lipid bilayer rearrangement during MP formation, and is a commonly used marker of MP in various studies [193, 274, 335, 575]. MAbs and protein were added sequentially to the appropriate wells in a 96-well round bottom plate (ThermoFisher Scientific Nunc™), in a darkened area, being careful not to mix them. Once complete, the plate was covered and placed in the cold room until use.

MP were prepared as per section 2.5 and stored on ice in 1 X AnV buffer, which is calcified, allowing the binding of AnV to phosphatidylserine [194]. To each well, 40µl of re-suspended MP were added and then incubated with the appropriate mAbs and AnV at room temperature, on a shaker and in the dark for 30 minutes. Once this step was over, the reaction was stopped using 200µl 1 X AnV buffer.

2.9.2 Sizing and enumerating microparticles

MP were enumerated and characterised on the flow cytometer using latex beads. MP were identified as < 1.1µm in diameter, using 1.1µm latex beads (Sigma Aldrich,

cat no. LB11). 2µl 1.1µm beads were diluted in 2mls molecular grade water. This was swirled gently to mix and then 10µl of this suspension was added to 240µl molecular grade water in a 5ml round bottom tube (BD Falcon™, cat no. 352053). This was run on the flow cytometer and a gate below the 1.1µm beads was set on the forward *versus* side scatter plot to identify MP by size.

Since there is variability in the flow rates of flow cytometers, which can occur both within the same flow cytometer (intra-machine variability), and between different flow cytometers (inter-machine variability), MP event counts require standardisation. A known concentration of 3µm latex beads (Sigma Aldrich, cat no. LB30) was used to standardise counts to enumerate MP. 3µm latex beads were used, because they were of a known concentration and were seen reliably and consistently in an area outside the normal gating MP, allowing for the enumeration of MP [576]. 6µl 3µm beads were added to 2mls molecular grade water and swirled gently to mix. 10µl of this 3µm bead suspension was added to 240µl molecular grade water and ran for 30 seconds on the flow cytometer. 3µm beads were identified on the forward *versus* side-scatter plot and a gate was put around it. This was the 3µm bead count and then subsequent MP event counts were calculated using the following formula:

$$MP\ count\ per\ ml = \frac{(200{,}000\ \div 3\mu m\ bead\ event\ count)\ \times MP\ event\ count}{mls\ of\ plasma}$$

The concentration of 3µm beads in the suspension was known to be 200,000 per ml. This allowed there to be a standard reference of how many MP there were per ml of plasma. The volume of plasma used was 40µl and the 3µm bead count within the gate was used, which tended to be 3 - 4000. The MP samples were run and the event count measured was multiplied by the ratio of the concentration of the 3µm bead suspension. The number of MP/ml of plasma was calculated by taking into

account the number of tubes and the total number of millilitres of plasma from which the tubes were taken.

2.9.3 Compensation

Compensation was performed using Versacomp antibody capture beads (Beckman Coulter, cat no. B22804), which bind all mouse isotypes. Compensation is important to make sure that there is separation between fluorochromes, in particular FITC from PE. This was prepared using the Versacomp beads, and the compensation matrix was set up for each antibody and combination.

2.10 Flow cytometry with anti-tissue factor pathway inhibitor K1 monoclonal antibody

The mAb to TFPI K1 domain (Sanquin, cat no. MW1846, clone CLB/TFPI Kunitz-1 domain specific) used in this assay was unconjugated. In view of the need to avoid multiple steps and washing of MP following the addition of mAbs, it was not feasible to use conjugated antigen binding fragments to conjugate this antibody. Therefore, another process was utilised to bind this antibody. Apex® molecular beads, which were bound to Alexa Fluor (AF) 647 fluorochrome (Life Technologies, cat no. A1045), were used. These beads provided a convenient method for covalently attaching a fluorochrome to small amounts (10 – 20µg) of antibody.

This antibody labelling kit utilized a solid-phase labelling technique that captured IgG antibody on the resin inside the APEX® antibody labelling tip, enclosed within the packaging. Initially contaminants were eluted through the tip and then the amine-reactive, fluorescent label, in this case AF647, was added to the IgG

antibody on the resin. This was incubated for 2 hours at room temperature in the dark, leading to the formation of a fluorescent IgG conjugate. The final step was to elute the conjugated antibody from the resin using elution buffer. The amount of antibody conjugated at a time was 20µg, with the volume of antibody at the end of the process being 50µl. The conjugated antibody was either used as above in flow cytometry or stored at 4˚C, for a maximum of 2 weeks.

2.11 Preparation of HUVEC and HUVEC-derived microparticles

Confirmation of the efficacy of the AF647 conjugated anti-TFPI K1 antibody was performed using human umbilical vein endothelial cells (HUVECs) and HUVECs induced MP. Human umbilical veins were collected from the obstetrics department as per prior agreement and validated by Imperial College Healthcare NHS Trust and Imperial College. Two media were used in preparing HUVECs and for their growth:

1. Complete Medium
    - 500ml Medium-199 (Sigma Aldrich, cat no. M2154)
    - 5ml L-glutamine (Sigma Aldrich, cat no. G7513)
    - 5ml Penicillin/Streptomycin (Sigma Aldrich, cat no. P0781)
    - 100ml heat-inactivated foetal calf serum (FCS) (20%) – batch tested (56˚C for 30 minutes in the waterbath)
    - 15mg endothelial cell growth factor (ECGF) (Sigma Aldrich, cat no. E9640) in heparin (30µg/ml)
2. Plating Medium
    - 500ml Medium-199
    - 5ml L-glutamine

- 50 ml heat inactivated FCS
- 3.75mg ECGF in heparin (7.5µg/ml)

All procedures were performed in sterile conditions in the tissue culture hood. 75 ml flasks were prepared by coating them with 5ml gelatine solution 2% (Sigma Aldrich, cat no. G1393-100ml), which had been heated for 30mins in a 37˚C waterbath. Following coating of the flask, excess gelatine was discarded using a 5ml pipette, and the flasks were capped and placed in the hood. The umbilical vein was cleaned using PBS, cells were flushed into a flask and then incubated in a 75ml flask using complete medium, under sterile conditions, being careful to avoid infection, at 37˚C.

Flasks were carefully checked using a microscope and once the cells appeared to be confluent, they were then collected and split. This is known as a 'passage'. When required, adhered cells were loosened from the flask using 1.5ml trypsin, which had been heated to 37˚C in a waterbath. This took 30 seconds, and once loosened, 13.5ml complete medium was added to the flask, so as to inactivate the trypsin, allowing collection of the cells. The cells were then washed by centrifuging them for 5 minutes at 300g, with deceleration set at 9. The supernatant was removed, and then 10mls PBS, heated to 37˚C in the waterbath, and then centrifuged at 300g for 5 minutes, with deceleration set at 9. The PBS was then removed, and the cells were resuspended back to 15ml, and then split. The cells were then added to a sterile 75ml flask coated with gelatine, and a further 7.5ml complete medium was added to give a total of 15ml. The flask was then incubated at 37˚C, with assessment of confluence made the next day.

Once at third or fourth passage, cells were collected as above, but rather then placing the cells in another flask, they were diluted in plating medium and plated out on a 6 well plate, at a concentration of 500,000 cells per well in 1 ml of plating medium. Cells were incubated for 24 hours, and then they were collected. Firstly,

the media was collected and centrifuged at 300g for 5 mins, with deceleration set at 9, to removed dead cells. Following this, the supernatant was removed and this supernatant contained HUVEC MP and was stored at -80˚C.

Following removal of the supernatant, 100µl trypsin was added to each well and left to loosen adhered cells for 30 seconds. Subsequently 900µl plating medium was added to each well to dilute trypsin, thereby inactivating it. The supernatant was then removed and the cells were collected after being resuspended in 1ml plating medium following scraping. Cells were stored in 1ml aliquots at -80˚C.

Once required, cellular samples were thawed at 37˚C in waterbath for 1 – 2 minutes and then processed accordingly. Cells were span for 300g x 5 mins to remove debris, and then at 700g x 5 mins to sediment cells. These were re-suspended to the original volume in PBS ready for flow cytometry. The HUVECs supernatants were centrifuged as previously described at 17,000g for 1 hour to sediment MP. This was then re-suspended to 500µl rather than 1ml and stored on ice ready for flow cytometry.

2.12 Coagulation lysis assay

This was a turbimetric assay which assessed clot formation and lysis, following the addition of a plasminogen activator, using absorption spectrophotometry. The assay was used to assess the procoagulant and lytic activity of each patient and HC sample PPP, MP and MPPP. These were run in quadruplicate on a 96-well flat bottom plate (ThermoFisher Scientific Nunc™) and the method was as follows.

After MP were prepared from PPP, as per section 2.6, they were re-suspended in sterile molecular grade water to their original volume of 500µl. 100µl of the MP suspension was added to each of the wells that were planned to assess the MP in a

96-well flat bottom plate. The other wells that were to be used to assess PPP and MPPP were filled with 100µl molecular grade water and the plate was then pre-incubated for 60mins at 37˚C to allow adherence of the MP to the well surface. MP are known to adhere to wells via phosphatidylserine [577].

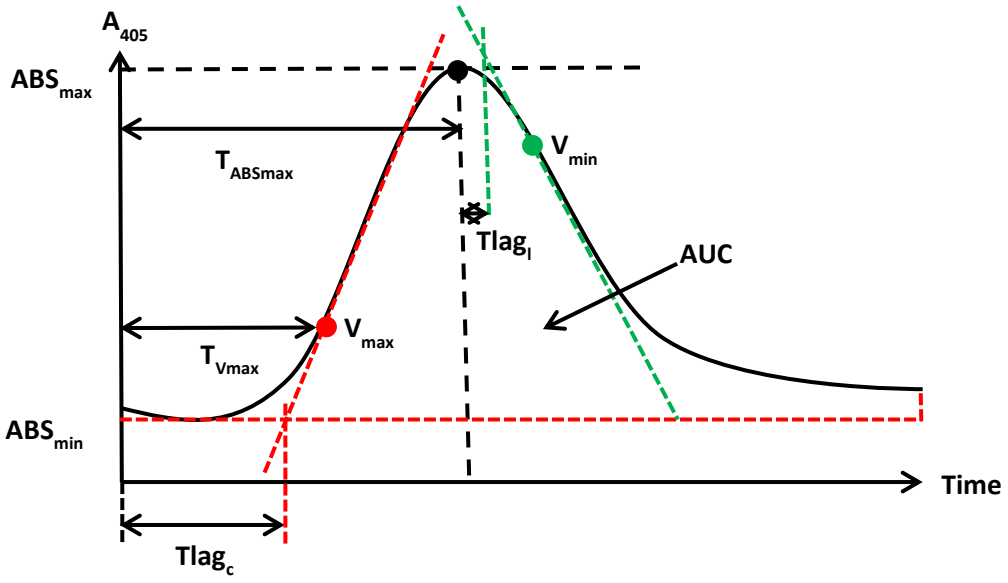
Whilst the plate was incubating, PPP, autologous MPPP and normal pooled MPPP were prepared containing the thrombolytic agent, reteplase (Activase, Rapilysin®). Reteplase is a recombinant serine protease tissue plasminogen activator that only operates in the presence of fibrin, so it can be added to plasma prior to clot initiation [578]. Reteplase was reconstituted from its powder with the enclosed solvent to give a total of 10U in a 10ml volume. It was stored as 50µl aliquots (50µU) at -80˚C ready for use. Dilution experiments were performed as per section 3 to determine the required reteplase concentration per 100µl of plasma to elicit lysis. Reteplase was added to the appropriate pooled plasma and gently mixed and stored on ice until required.

Once the pre-incubation step was complete, supernatant was removed from the wells. 2.5µl of 1M $CaCl_2$ was added to each well, following which the appropriate plasma containing reteplase was added to each well. The plasma added to the MP wells was either pooled autologous MPPP or normal pooled MPPP. This allowed comparison between various plasma and measured the activity of adhered MP compared to PPP. The plasma was added rapidly using a multichannel pipette, so that the coagulation process did not begin prior to the plate being read.

The plate was placed in the spectrophotometer (Synergy HT, Biotek, Fisher Scientific) at 37ºC and clot turbidity was measured at an absorption wavelength 405nm (A_{405}) every minute for 60 minutes. Figure 2-2 shows the number of different measures that can be made. These include the clot lag time ($Tlag_c$), which is the

time to exponential clot formation and clot lysis time ($Tlag_l$), which is the time to exponential lysis. Exponential clot formation is the point of the curve when absorption first increases above baseline, as the clot first forms. Exponential lysis is the point of the curve following peak absorption when absorption first declines, as lysis first begins. Other measures include V_{MAX}, which is the speed of maximum clot formation, and the area under the curve (AUC). The results were analysed following modelling using a quadratic equation in Table Curve 2D v 5.01 (Systat software, San Jose, USA). This allowed smoothing out of curves, so that the above measures could be taken.

Figure 2-2 Coagulation lysis curve



This is a representation of the coagulation lysis curve obtained following the turbimetric assay. A number of different measurements can be made including the clot lag time ($Tlag_c$), clot lysis time ($Tlag_l$), maximum velocity (V_{max}), which is the maximum speed of clot formation, and area under the curve (AUC). The Tlagc is the time to exponential clot formation, which is the point of the curve when the absorption first increases as the clot forms. Similarly, the $Tlag_l$ is the time from peak absorption to exponential lysis, which is the point of the curve when the absortion first declines after the peak, representing the beginning of lysis. Other labels: ABS_{max} = maximum absorption; T_{ABSmax} = time to maximum absorption; T_{Vmax} = time to maximum velocity; ABS_{min} = minimum absorption; V_{min} = minimum velocity, which represents the most rapid phase of lysis.

2.13 Calibrated automated thrombin generation assay

The CAT assay is a well-recognised assay to assess the procoagulant properties of plasma and has been utilised to identify the procoagulant function of MP [262, 301-303]. This assay was used to identify the procoagulant properties of MP, prepared as per section 2.6, from healthy controls and BS patients. The method utilised the intrinsic procoagulant properties of MP, so no phospholipids (PLs) or exogenous tissue factor (TF) were added to these samples. However, controls utilised PLs and exogenous TF. Reagents used were:

- DOPE (phosphatidylethanolamine), DOPC (phosphatidylcholine), DOPS (phosphatidylserine) synthetic phospholipids (Avanti® Polar Lipids, cat no. 850725C and 790595P)
- Tris (hydroxymethyl) aminomethane (Sigma Aldrich cat no. 252859)
- Corn trypsin inhibitor (CTI) (Haematologic Technologies Inc., cat no. CTI-01)
- Calcium chloride dihydrate (Sigma Aldrich, cat no. C-3881)
- Bovine serum albumin (Sigma Aldrich, cat no. A3803)
- Sodium chloride (Sigma Aldrich, cat no. S9625)
- Innovin® recombinant TF (Siemens, cat no. 539179)

Firstly, 1M Tris buffered saline was made using 6.05g Tris and 8.76g sodium chloride in 800ml ddH_2O. The pH of the solution was adjusted to 7.4 using 1M hydrochloric acid. Once done, ddH_2O was added to make the volume 1L. This was then stored at room temperature ready for use. From this 50ml 20mM TBS was made by adding 1ml 1M TBS to 49ml ddH_2O.

The stock concentration of PL utilised was 1mg/ml, equivalent to 1,250μM. The combination of PLs were 60% DOPC, 20% DOPS and 20% DOPE to make up a total volume of 100μl. These were added together quickly so they did not evaporate,

since they were stored in chloroform. As both PLs are photosensitive, they were prepared in a dark bottle. The PL mix was dried with nitrogen gas, which speeded up the drying process. This was important since it takes 4 hours for the PL mix to dry out in room air, and PLs oxidise with time. The PL mix was dried for 1 minute longer than required, leaving a pale film on the inside of the bottom. 1ml 20mM TBS was added to the bottle, and the PLs were dissolved in this by vortexing the bottle for 4 minutes, creating a milky solution.

The solution was then extruded to remove any clumped PL particles. The extruder was set up by placing a 0.1µm filter in between the two halves, then 1ml of 20mM TBS was passed through to remove the air from it. The glass syringes were connected to both sides of the extruder, with one syringe containing the 1ml PL solution. The PL solution was then passed through the extruder 19 times, leaving it clear. It was then transferred into a 1.5ml Eppendorf, covered in aluminium foil and stored at 4˚C. PLs were used within five days or were discarded.

Further buffers used in this assay included:

1. 20mM TBS and 0.5% BSA buffer

   To make 50mls, 1ml 1M TBS was added to 49mls ddH_2O, to which 250mg BSA was also added. This was shaken carefully, to make sure the BSA dissolved and making sure the solution was not too 'frothy'.
2. 60mg/ml BSA in 20mM TBS

   3mls of this buffer was made by adding 180mg BSA to 3mls of 20mM TBS. This was then filtered using a syringe and 0.45µm filter. Subsequently, 1.75ml of this solution was transferred into a clear glass bottle and 200µl 1M calcium chloride was added to this, giving a total volume of 1.95mls. This was then preheated in a water bath set at 37˚C in preparation for the final step

The MP were prepared as per methods section 2.6, except instead of being re-suspending in 1 X AnV buffer, they were re-suspended in 20mM TBS using the equivalent MP in 500µl PPP in each well. Therefore, a fixed number of MP were not studied, but MP from a fixed volume of PPP from each participant.

Pooled PPP came from 10 normal healthy controls. When MP were used, the pooled healthy control PPP was centrifuged as per normal protocol to derive MP, and the MP pellet was removed leaving MPPP. This was used in place of PPP in the samples with MP and in those where the MPPP was used on its own. This is shown below:

MP + NMPPP	**MPPP**	**PPP**
1. 80µl MPPP	1. 80µl MPPP	1. 80µl PPP
2. 10µl MP suspension	2. 10µl 20mM TBS and 0.5% BSA buffer	2. 10µl 20mM TBS and 0.5% BSA buffer
3. 10µl 20mM buffer	*3.* 10µl 20mM buffer	*3.* 10µl 20mM buffer

The final PL concentration in the controls was 4µM, whilst the original stock PL concentration was 1,250µM. In the controls, the final PL volume was 5µl out of a total volume of 120µl. A further dilution of the PL stock solution was required from which the 5µl was taken from. The concentration of this PL solution was calculated to be:

$$C1\,V1 = C2\,V2$$

$$4 \times 120 = C2 \times 5$$

$$480 = C2 \times 5$$

$$480 \div 5 = C2$$

$$96\mu\mathrm{M} = C2$$

The volume of stock PL solution required to make a 20µl 96µM PL solution was:

$$C1\,V1 = C2\,V2$$

$$96 \times 20 = 1250 \times V2$$

$$1920 = 1250 \times V2$$

$$1920 \div 1250 = V2$$

$$1.536\mu L = V2$$

This was then added to 18.5µl 20mM TBS + 0.5% BSA.

The original TF concentration in Innovin® is 6000pM from which a 500pM stock solution following dilution in ddH_2O was done. There were two levels of TF controls, one that was 1pM and the other 4pM. Other concentrations were used for setting up experiments, and the calculations were made accordingly. 5µl of each concentration was required in the final 120µl volume, so the concentrations of TF solutions from which the 5µl was taken were:

1pM TF

$$C1\,V1 = C2\,V2$$

$$1 \times 120 = C2 \times 5$$

$$120 = C2 \times 5$$

$$\frac{120}{5} = C2$$

$$24pM = C2$$

4pM TF

$$C1\,V1 = C2\,V2$$

$$4 \times 120 = C2 \times 5$$

$$480 = C2 \times 5$$

$$\frac{480}{5} = C2$$

$$96pM = C2$$

From the original 500pM TF solution, 30µl 24 and 96pM TF solutions were required. The volumes required from the 500pM TF solutions were:

1pM TF

$$C1\,V1 = C2\,V2$$

4pM TF

$$C1\,V1 = C2\,V2$$

$$24 \times 30 = 500 \times V2$$

$$720 = 500 \times V2$$

$$\frac{720}{500} = V2$$

$$1.44\mu l = V2$$

$$96 \times 30 = 500 \times V2$$

$$2880 = 500 \times V2$$

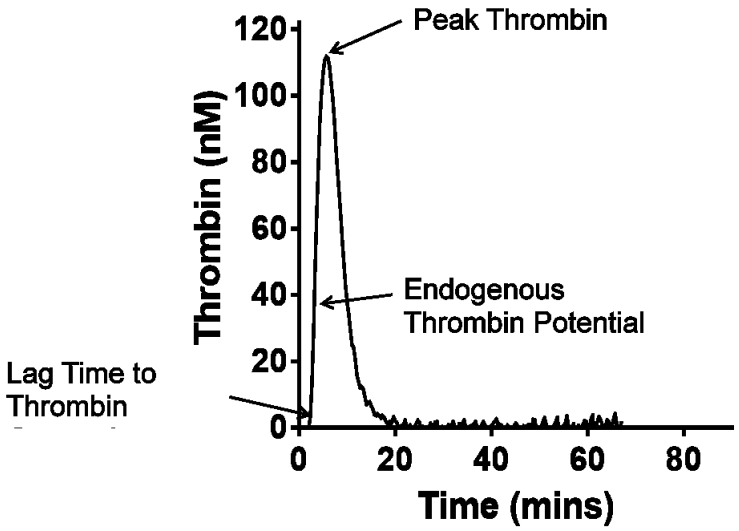
$$\frac{2880}{500} = V2$$

$$5.76\mu l = V2$$

Add 28.56µl and 24.24µl 20mM TBS + 0.5% BSA were added to create the 30µl 24 and 96pM TF solutions respectively. In each assay, a thrombin calibrator (Thrombinoscope, cat no. TS20.00) was used with the appropriate PPP or MPPP. The Thrombin Calibrator was used for each individual plasma to correct for donor-to-donor differences in colour of plasma, inner filter effect and substrate consumption.

The samples were run on the Fluoroskan Ascent FL plate reader (Thermo Lab system) in combination with Thrombinoscope software (Synapse BV) as previously described. Thrombin generation was monitored using 0.42mM of the fluorogenic substrate Z-GlyArg-AMC-HCl (Bachem), which had been diluted in 60mg/ml BSA in 20mM TBS with calcium. The final concentration of calcium in each sample was 16.6mM, which allowed initiation of thrombin generation. The measures were taken at 460nm fluorescence, every minute for 90 minutes. From these then the below measures could be taken:

Figure 2-3 Thrombin generation curve from the calibrated automated thrombin generation assay



This a typical thrombin generation curve from the CAT assay. Endogenous thrombin potential (ETP), represents the area under the curve, which is the total thrombin generated, whilst peak thrombin and lag time to thrombin generation ($Tlag_c$) can also be measured.

Endogenous thrombin potential, ETP, is the area under the curve and is the total thrombin generated. These were then compared to MP numbers as calculated using flow cytometry.

2.14 Zymuphen MP-TF activity assay

The Zymuphen MP-TF (Hyphen Biomed, Aniara cat no. A521196) measures MP TF function via the production of FXa. The assay is a capture ELISA whereby, the plate wells contain a non-inhibitory antibody to TF, to which TF+ MP bind. The assay was performed as per manufacturer's instruction, except for the preparation of PPP, which was as for method section 2.4. Once PPP samples were thawed, they were stored on ice and samples were used within 1 – 2 hours of thawing.

Firstly, 200µL buffer and 20µl sample, control or standard were added to each well, containing a non-inhibitory antibody to TF. TF+ MP present in the sample bound to

the solid phase through an epitope localized in the extracellular domain of TF. The samples were then incubated at room temperature overnight and subsequently underwent the first washing step, to clear excess plasma.

Factor VIIa and Factor X were added, which led to the formation of the TF-FVIIa complex to convert Factor X into Factor Xa, on the surface of the anionic phospholipids present on MP, and in presence of calcium ions. The amount of Factor Xa generated is proportional to the amount to TF+ MP. In the final step, a specific substrate of Factor Xa is added to the sample to measure the amount of Factor Xa generated. This substrate is cleaved leading to yellow discolouration of the wells, and after 10 minutes the sample are read at 405nm wavelength in the spectrophotometer. A standard curve was set up with known concentrations of TF, so that the amount of TF activity present could be calculated in pg/ml.

2.15 Statistics

Results of all the assays followed a non-Gaussian distribution, so are expressed as medians. GraphPad Prism 6.07 (GraphPad Software, Inc, San Diego, CA, USA) was used for statistical analyses and graph generation. Statistical comparisons of median MP counts, MP proportions and CAT assay measures were accomplished using Mann-Whitney U test with significance set at $p < 0.05$.

3 Optimisation of Microparticle Flow Cytometry

3.1 Background

MP are anuclear buddings of cells undergoing activation or apoptosis. They tend to be 100 – 1000 nm in diameter and preferentially express PS on their surface. This is the result of their production, which involves lipid bilayer rearrangement as a result of the phospholipids moving down concentration gradients. This is promoted by a series of surface transmembrane proteins, nominally called ‘flippases’, ‘floppases’ and ‘scramblases’.

Along with sizing, surface PS is a hallmark of MP and binds to AnV, which makes it a useful marker to identify MP when performing flow cytometry, particularly in the context of investigating their procoagulant properties [579]. On release, MP retain the surface markers of their parent cells, such as monocytes (CD14 and TF), platelets (CD41a and CD62P) and neutrophils (CD14 and CD66b). There has been extensive use of these surface markers published in the literature to identify these MP sub-types in many conditions [193, 321, 344, 580-582].

The ability to detect MP by size and surface characteristics has led to the common use of flow cytometry as a modality to identify them. By using multiple mAbs, one is able to further elucidate the MP population, such that MP can be dual or triple stained, although there is concern that use of multiple mAbs lead to steric interference, due to the size of fluorochromes compared to the size of particle [583]. Usually, the size fluorochrome rather than the mAb causes this problem, and although groups do not report steric interference as a particular concern, few use more than 3 mAbs to stain MP.

Flow cytometry is a powerful modality to identify MP, but, although there are efforts being made in this area, there are no standardised protocols, [216, 257, 268, 271].

This even goes back to sample preparation, resulting in a wide variation of centrifugation speeds for preparation of platelet poor plasma and MP preparation, time between sample collection and processing, freeze-thawing and use of citrate versus EDTA as the anticoagulant of choice.

Much of the lack of standardisation is due to the variability between flow cytometers. Many flow cytometers were not designed with the detection of MP in mind, and so attempts have been made to standardise protocols on a particular machine [268]. Clearly, this is unfeasible as centres will have different flow cytometers, and there are striking differences between flow cytometers, with newer models resolving smaller particles with less background than older machines [244].

Cleanliness of the system is imperative, since the threshold is often set very low to measure tiny particles and much of cell flow cytometry is based around higher thresholds to avoid this background noise. As a result, it is important to make sure that reagents used are clean, in particular sheath fluid and diluents. Some groups advocate lengthy preparation and cleaning times, even going so far to filter sheath fluid, as well as other buffers prior to running samples [279]. Whether this should be done across the board is debatable, since elaborate cleaning regimens may make MP flow cytometry laborious and impracticable for mainstream testing.

There have been few studies of MP in BS. CD62P+ MP have been shown to be increased in BS, compared to healthy controls [346]. A proprietary MP functional assay, which identifies MP by capture ELISA, showed increase levels of MP in BS, but this did not align with any particular clinical manifestations of BS [347]. This suggests there remains scope for analysis of MP in BS, especially in developing markers to identify particular manifestations of BS, such as thrombosis.

Herein, I elected to optimise a MP flow cytometry protocol to identify total AnV+ MPs and cellular subsets using mAbs to CD14, CD66b and CD62p. I elected to base my protocol upon that developed by Dr. Paul Brogan's research group, University College London, who have extensive experience in this area [343, 344, 584].

3.2 Methods and Materials

3.2.1 Peripheral blood samples

Samples were collected from healthy controls and BS patients recruited into the study as per sections 2.2 and 2.3. Within 1 – 2 hours of collection, samples underwent serial centrifugation to prepare platelet poor plasma (PPP) and were stored as 500µl aliquots at -80˚C awaiting analysis (see section 2.5 for further details).

3.2.2 Microparticle preparation

MP were prepared from PPP samples as per section 2.6. Briefly, samples were thawed at 37˚C and underwent centrifugation at 17,000g for 60 minutes. Following decanting of 480µl supernatant, the 20µl containing the MP pellet was resuspended in to 500µl in 1 X AnV buffer and stored on ice ready for mAb staining and flow cytometry.

3.2.3 Monocyte isolation

Monocytes were prepared from healthy control samples as detailed in section 2.7. Following preparation, monocytes were plated out into 6 well plates and underwent

a LPS time course, after which the supernatant was collected, centrifuged at 300g for 5 mins to remove cellular debris and stored in 1 ml aliquots at -80˚C. From these, MP were prepared as per section 2.6.

3.2.4 Flow Cytometry

3.2.4.1 Sample staining

Flow cytometry is detailed in sections 2.8 and 2.9. Briefly 40µl MP suspension were stained with AnV and the appropriate mAbs: fluoroisothiocyanate (FITC) conjugated anti- TF, phycoerythrin (PE) conjugated anti-CD14, PE conjugated anti-CD66b, PE conjugated anti-CD62P and allophycocyanin (APC) conjugated anti-PSGL1. Controls were as documented, including unstained sample, matched isotype controls and 1 X AnV buffer. Samples were prepared as described.

3.2.4.2 Compensation

Versacomp Beads recognising the appropriate mAbs, including isotype controls, were used to assist with compensation and set up of gating (see section 2.9.3).

3.2.4.3 Sizing and enumerating beads

Prior to all sample runs, 1.1 and 3µm beads were used to size and enumerate MP respectively (see section 2.9.2). MP counts were calculated as per the equation given in section 2.9.2.

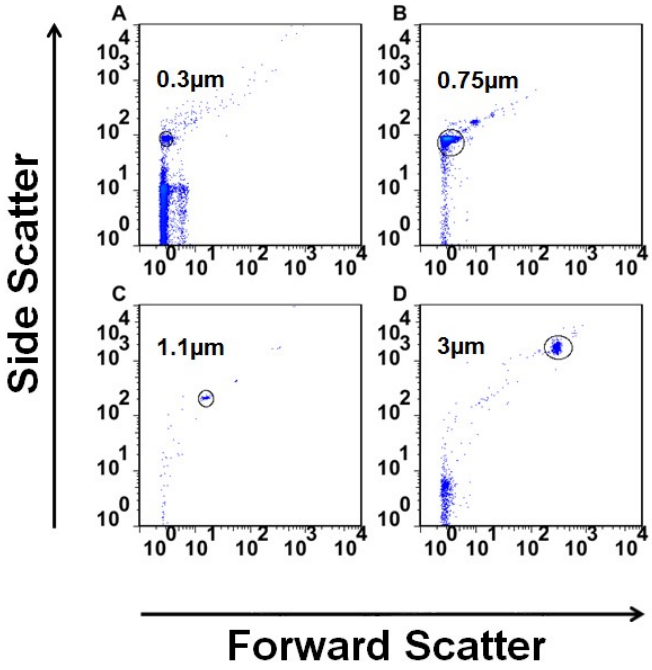
3.3 Results

3.3.1 Initial set up to visualise latex beads in dot plots

1.1µm and 3µm beads were used to size and enumerate MP, as documented in section 2.9.2. PMT voltages in forward and side-scatter were altered accordingly, such that they were visualised in the centre of the histogram, with threshold set to accommodate the beads. A threshold of 5% on forward scatter did not visualise the beads, and so the threshold was dropped to 0.02% for the beads to be visualised. This threshold setting is unique to Beckmann Coulter Cyan ADP flow cytometer, with the lowest threshold setting being 0.01%. It was elected to continue to threshold on forward scatter rather than fluorescence, to make sure all the fluorescent MP in the gate below 1.1µm were included.

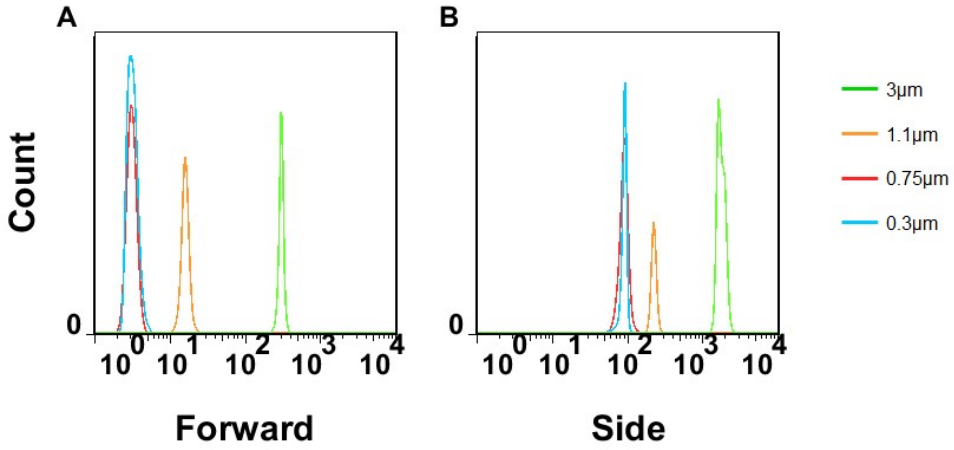
Figures 3-1 A – D shows the dot plots of 0.3µm, 0.75µm, 1.1µm and 3µm beads. These beads were resuspended in molecular grade water, and these were run over 30 seconds. Figure 3-1 shows the dot plots of the beads in size order. There was discrimination between 1.1µm and 3µm beads in forward and side scatter, but there was poor discrimination between 0.3µm and 0.75µm beads. In Figures 3-2 A and B, histograms show the position of the beads in both forward and side scatter. This shows that neither forward nor side scatter was able to resolve 0.3µm and 0.75µm beads.

Figure 3-1 Position of the latex sizing beads on the side *versus* forward scatter plots



Latex beads of diverse sizes were suspended in molecular grade water and run through the flow cytometer. The position of the beads on the side *versus* forward scatter plots are shown: **(A)** 0.3µm, **(B)** 0.75µm, **(C)** 1.1µm and **(D)** 3µm beads. There was little discrimination between the position of the 0.3 and 0.75µm beads. 1.1µm and 3µm beads were taken forward for sizing and enumerating.

Figure 3-2 Beads histogram overlay



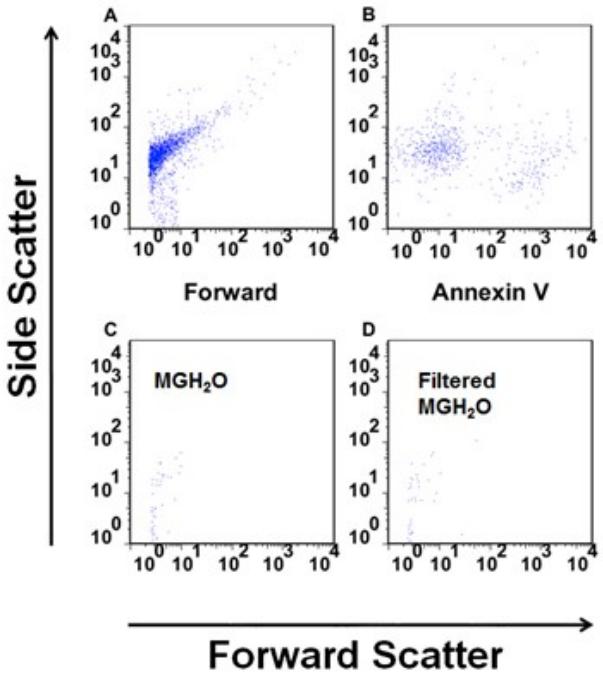
Comparison of latex beads separation using forward **(A)** and side-scatter **(B)**. There was clear separation between 1.1µm and 3µm beads in both forward and side scatter. There was no discrimination between 0.3µm and 0.75µm beads.

These results show that the flow cytometer was able to identify particles less than 1.1µm in diameter, but was unable to discriminate the specific size. This is typical of many flow cytometers, which are often unable to discriminate the size of particles below 0.5µm in diameter [244].

3.3.2 Cleanliness of the flow cytometer

It was important that the flow cytometer was kept clean at all times. The standard cleaning protocol was instituted at shutdown and prior to running of samples. Figures 3-3 A and B shows a run in forward *versus* side scatter and in side scatter *versus* AnV with an unclean machine. Debris was located within the <1.1µm gate (Figure 3-3 A) and there was autofluorescence within the side scatter *versus* AnV dot plot (Figure 3-3 B). This shows that without keeping the machine clean results could be significantly impacted.

Figure 3-3 Background noise and cleanliness of the flow cytometer with runs of molecular grade water



These plots show the importance of keeping the flow cytometer clean. **(A)** shows there is considerable background noise within the standard gate set for particles < 1.1µm in diameter in the forward *versus* side scatter, which would include MP, in an unclean flow cytometer. **(B)** shows the autofluorescence of these debris in side scatter *versus* AnV plot. **(C)** and **(D)** are runs of unfiltered and filtered MGH_2O through the flow cytometer. These were run after cleaning with Beckmann Coulter Coulter Clenz® and MGH_2O for 20 minutes each. This shows that with both unfiltered and filtered MGH_2O, there were < 50 event counts in the plot. There was no difference made by filtering the MGH_2O. MGH_2O = molecular grade water.

Standard measures were used to keep the machine clean, so Beckmann Coulter Coulter Clenz® and molecular grade water (MGH_2O) were used to clean the machine. Before each individual sample, Coulter Clenz® was run for 1 minute on high flow sample clean setting of the flow cytometer. Then Coulter Clenz® and MGH_2O was run at high flow for 20 minutes each to make sure the machine was clean. Following these runs, MGH_2O was run to assess the background noise in the forward *versus* side scatter plot. In Figures 3-3 C and D, comparison was made between unfiltered and filtered MGH_2O using a 20µm filter. There was no difference between the two samples, with total events in the gate < 50. These were run before every sample and samples were only run once the event count was < 50. It has been shown that the fluidics can provide background noise, which can impact on the

measurement of MP [244]. In this case, the sheath fluid in the fluidics was ddH_2O and 1% bleach, which did not show background noise based upon Figures 3-3 C and D.

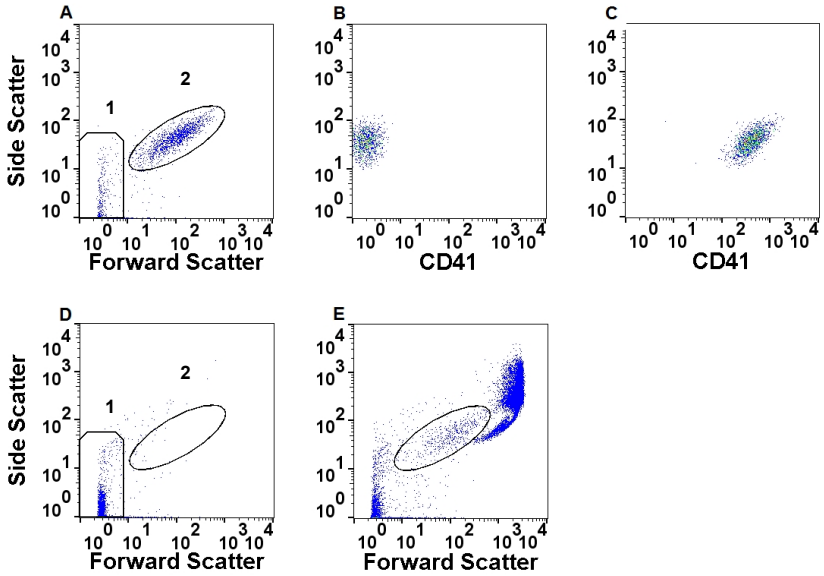
3.3.2.1 Confirmation that platelet poor plasma samples were truly platelet poor

In this study, MP were prepared from PPP, so it was important to establish that PPP samples were truly platelet poor. It is important that PPP are platelet poor to make sure that there is no further MP production from activated platelets, which would affect the results. In Figure 3-4, PPP were compared with autologous PRP and whole blood. PPP and PRP were prepared as per section 2.5. PPP were run undiluted, whilst PRP was diluted 1:100 in MGH_2O. Whole blood was diluted 1:1000 in MGH_2O before being run.

Figures 3-4 A – C show PRP. Figure 3-4 A shows a side *versus* forward scatter plot of PRP. The side *versus* forward scatter plot was the same set to identify MP, with the less than 1.1µm bead gate. The circled population is platelets. Figures 3-4 B and C show side scatter *versus* CD4l plot. The mAb used was an APC-conjugated anti-CD41 mAb (Biolegend Cat no. 303710), which was used at a concentration of 1ng/µl used in Figure 3-4 C. The isotype control was a APC-conjugated murine IgG1κ mAb (Biolegend Cat no. 400120), which was used at the same concentration as the anti-CD41mAb. PRP was incubated with the isotype and mAb for 30 minutes at 4˚C in the dark. Figure 3-4 B shows platelets that were in the platelet gate established in Figure 3-4 A stained with the isotype control. Figure 3-4 C shows platelets that were stained with anti-CD42b mAb. The platelets positively stained for the anti-CD42b mAb confirming their origin. Figure 3-4 D shows PPP in side *versus* forward scatter plot. Within the platelet gate established in Figure 3-4 A there were

very few events. Figure 3-4 E shows whole blood in the side *versus* forward scatter plot and there were platelets in the platelet gate, with cells to the left of the dot plot. These results show that PPP prepared by the method as described in section 2.5 is platelet poor.

Figure 3-4 Flow cytometry of autologous PPP, PRP and whole blood to establish that PPP was truly platelet poor



Flow cytometry was performed on autologous PPP, PRP and whole blood. PPP samples were assayed undiluted, whilst PRP and whole blood were diluted in MGH_2O at 1:100 and 1:1000 dilutions respectively. 1 is the MP gate (< 1.1μm). **(A)** Platelets from PRP were identified in the side *versus* forward scatter plot and a gate was drawn around them (2). **(B,C)** shows side *versus* CD41 plot. PRP were stained with a FITC-conjugated anti-CD42b mAb and isotype. **(B)** shows PRP incubated with the isotype, whilst **(C)** shows positive staining of the platelets with CD41 mAb compared with the isotype. **(D)** side *versus* forward scatter plot of PPP showed very few events in the platelet gate, whilst there were platelets seen in the whole blood sample **(E)**.

3.3.3 Titration of annexin V and monoclonal antibodies

3.3.3.1 Microparticle preparation from lipopolysaccharide stimulated whole blood

In this thesis, MP were identified by size < 1.1μm and binding of AnV due to preferential surface expression of phosphatidylserine. To identify appropriate concentrations of AnV and mAbs to cell surface markers, a source of MP was

required. The source of MP came from whole blood stimulated with LPS. Whole blood was collected as per section 2.4 and then stimulated with LPS at a concentration of 10ng/ml. The blood was incubated with LPS at 37˚C for 4 hours. Following this step, PPP was prepared, aliquoted and stored as per section 2.5. MP was prepared from this PPP, which I will term 'LPS stimulated PPP' from hereon.

3.3.3.2 Pacific blue annexin V concentration to identify microparticles

A tritration series was set to identify the appropriate final concentration of AnV, Figure 3-4 A – H shows the increasing AnV concentration in the final assay volume of 250 µl. A concentration of 1:500 identified MP (Figure 3-4 G).

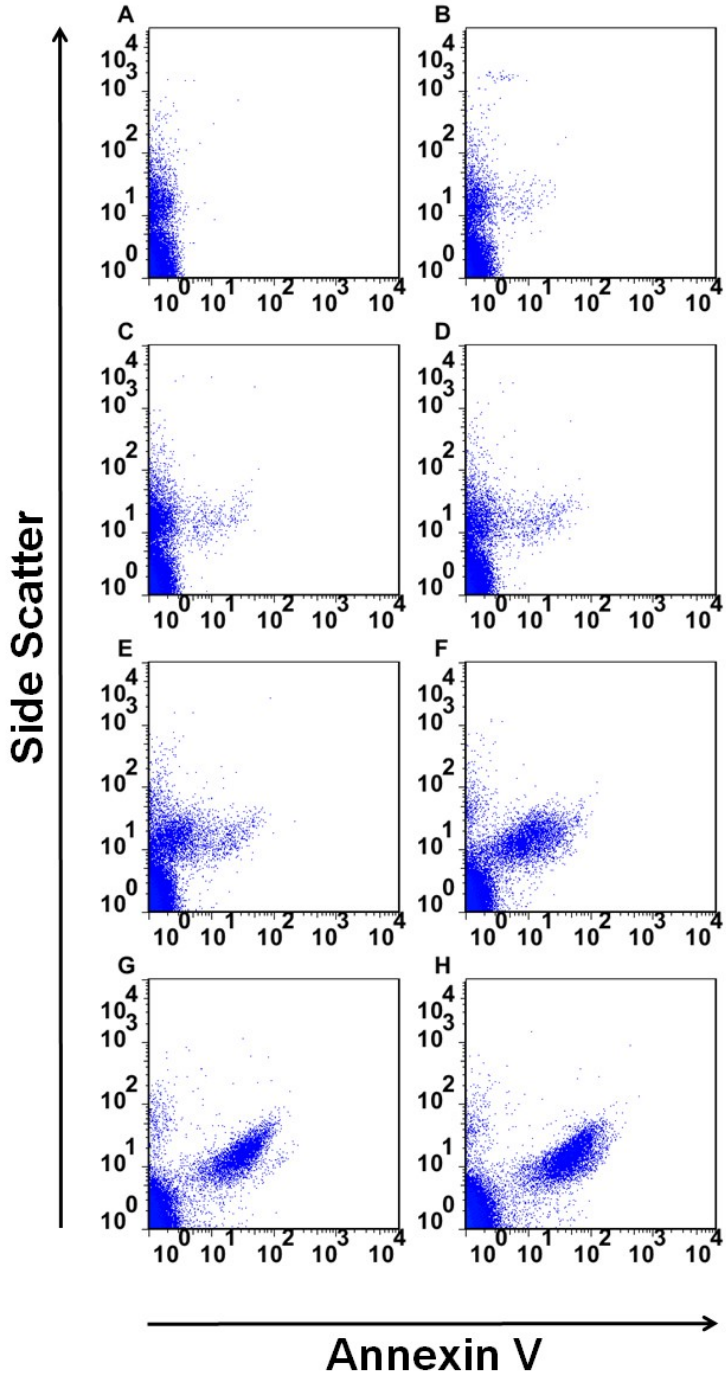
3.3.3.3 Optimisation of antibodies binding cell-derived microparticle

mAbs to CD14 (monocytes), CD62P (P-selectin, activated platelets and endothelial cells), CD66b (neutrophils). TF and PSGL-1 underwent titration series, with the final concentration of mAb per µl end assay volume was calculated. Figure 3-6 shows an example of a titration series using the anti-CD62P mAb. Table 3-1 shows the final concentration of mAbs used with the associated isotype control.

Table 3-1 Final concentration of monoclonal antibodies

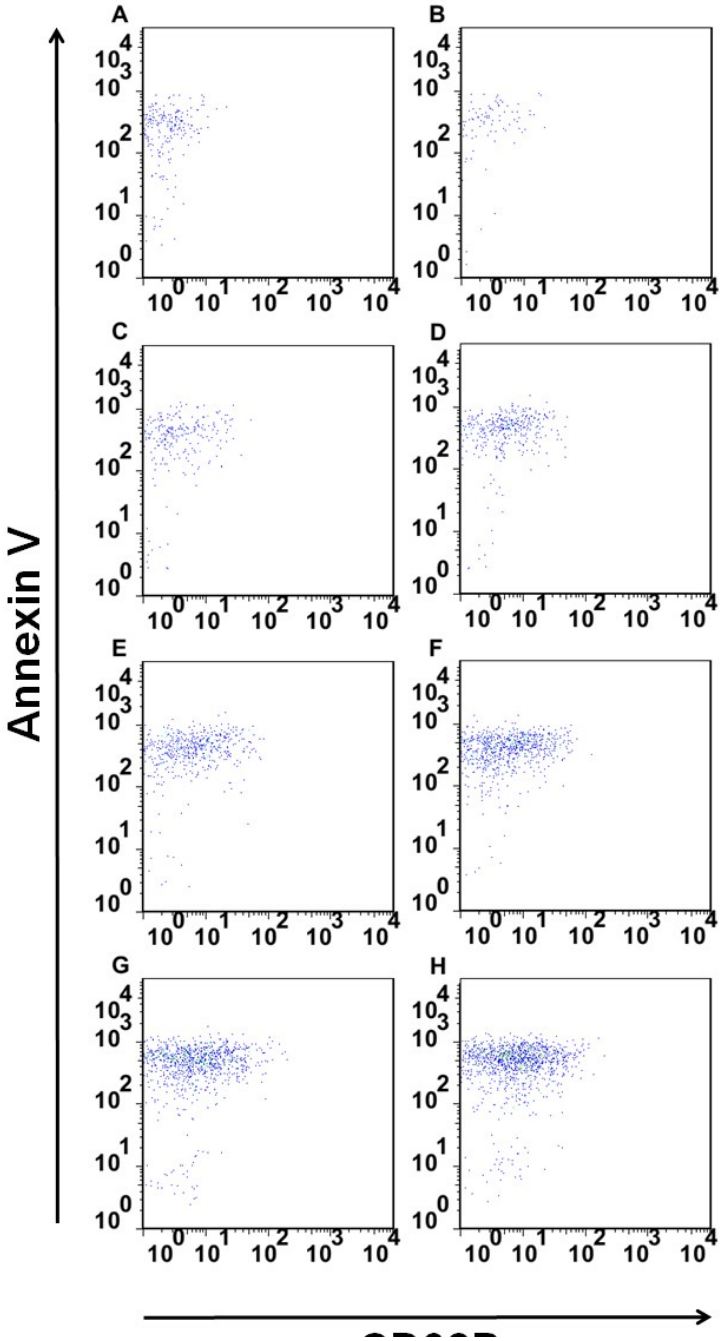
Monoclonal antibodies	Concentration (ng/µl)	Isotype
CD14	0.4	IgG2a
CD66b	0.4	IgG1
CD62P	0.4	IgM_{κ}
TF	0.8	IgG1
PSGL-1	0.2	IgG2a

Figure 3-5 Annexin V Pacific Blue titration series



AnV Pacific Blue titration series. Final concentrations in a 250µl assay volume for MP flow cytometry were: Unstained **(A)**, 1:25,000 **(B)**, 1:10,000 **(C)**, 1:5,000 **(D)**, 1:2,500 **(E)**, 1:1000 **(F)**, 1:500 **(G)** and 1:250 **(H)**. There was separation of the AnV population from the background at a concentration of 1:500. This concentration of AnV pacific blue was used in all the assays.

Figure 3-6 PE-conjugated anti-CD62P monoclonal antibody titration series

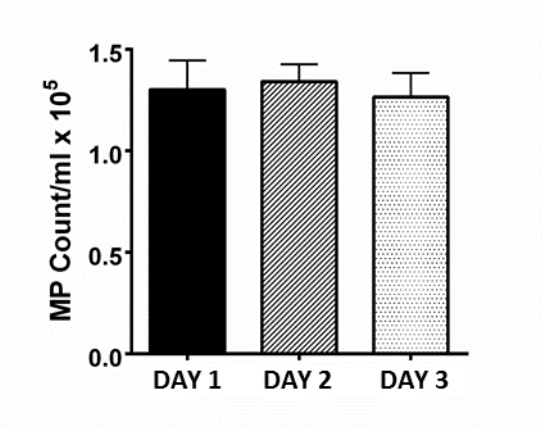


PE-conjugated anti-CD62P mAb titration series: 0.004 ng/μl **(A)**, 0.008ng/μl **(B)**, 0.016ng/μl **(C)**, 0.04ng/μl **(D)**, 0.08ng/μl **(E)**, 0.16ng/μl **(F)**, 0.4ng/μl **(G)** and 0.8ng/μl **(H)**.

3.3.4 Consistency of plasma cell membrane derived microparticle counts

MP were derived from pooled normal PPP from 10 healthy individuals as per section 2.6. These were run to assess the consistency of counts through the runs. MP were identified by sizing < 1.1μm and positive binding to Pacific Blue conjugated AnV, with total counts assessed. Concomitant use of 3μm beads standardised counts of MP, which were given as count/ml plasma. MP were prepared from the same PPP on three different days at separate times and run five times on each day, with results presented as mean count ± standard deviation. There was little difference in MP count between days 1,2 and 3 with the coefficient of variation 8.9%. This confirms that there was consistency across the runs.

Figure 3-7 Reproducibility of the microparticle assay



MP were prepared from normal pooled PPP and run sequentially five times on three different days at three separate times. Results are presented as mean ± standard deviation (SD). The coefficient of variation was 8.9%, showing that there was consistency between runs.

3.4 Discussion

3.4.1 Summary of results

Using the Beckmann Coulter Cyan® ADP flow cytometer I could detect MP that were less than 1.1μm in diameter and positive binding of fluorochrome-conjugated AnV.

Accurate size determination was not possible. Figure 3-2, showed that the flow cytometer was unable to resolve 0.3μm and 0.75μm beads either in side scatter or forward scatter. AnV and mAbs were titrated to find the most appropriate concentrations to identify MP and their cell surface expression. The MP were prepared from LPS stimulated PPP.

Other groups recommend filtering of sheath fluid and buffers, but I found a very low background when using unfiltered molecular grade water and 1 X AnV buffer, when the threshold was set at 0.02% on forward scatter. There was no additional noise from the sheath fluid, which was ddH_2O and 1% bleach. It was important to keep the flow cytometer clean. Running Beckmann Coulter Coulter Clenz® and molecular grade water between tubes to maintain cleanliness, reduced the background noise (Figure 3-3). This assisted with maintaining consistency of measure, with MP suspension from pooled healthy individual PPP providing MP counts with a low coefficient of variation over three consecutive days (Figure 3-7).

3.4.2 Reduction of background is important

Flow cytometers tend to detect particles less than 500 nm inconsistently [216, 268]. Comparison between machines is difficult, since some machines resolve MP more consistently using forward scatter, whilst others are better with side scatter [271]. Background remains a significant problem, but that also varies with sample preparation. Some groups advocate the use of whole blood MP analysis [585, 586], but this can lead to a number of problems. These include the need to analyse samples within 30 minutes and the significant background. The latter makes gating appropriate MP populations difficult, and as there is a need to analyse samples rapidly, batch testing is unfeasible. With the above protocol, I was able to collect

samples over a short period of time, and run large numbers together with the appropriate controls.

3.4.3 Sample preparation

The protocol used for sample preparation, was from a published protocol used as part of a continuing collaboration [204, 343, 344, 587]. Centrifugation speeds do vary from group to groups and there is concern that the yield of MP is much reduced with increasing speeds [216, 218, 244]. I have shown in Figure 3-4 that we were measuring with PPP. That is important, because of risk of platelet activation, which leads to increasing MP counts [193, 217, 254].

As discussed earlier in section 1.2.3.2, there are several disadvantages with measuring MP using the technique in this study. There is a reduction in MP, as result of serial centrifugation, there can be shearing of MP, although that tends to be at higher speeds, and there can be formation of microaggregates when sedimenting MP from PPP [221, 227, 233, 239, 249, 588]. Therefore, there are groups that recommend analysis directly from PPP, but this may lead to issues with using certain stains when assessing MP.

There is some concern that storing PPP at -80˚C can lead to increase in MP and degradation of sample. It has been shown that freeze thawing MP made little difference to their procoagulant function [317], whilst others have shown donor to donor variation in MP tissue factor activity [250]. The standard recommendation will biological samples is to avoid multiple freeze thaw cycles. This was avoided and samples only underwent a freeze thaw cycle once.

3.4.4 Use of annexin V

Expert opinion from Dr Paul Brogan's group recommended using AnV as the surface marker. AnV binds to PS, which is preferentially expressed on MP surface. PS also provides a negatively charged surface for coagulation factors to bind to, such that it is also a marker of procoagulant particles. As the focus of this thesis is thrombosis in BS and the possible role of MP in this, it seemed sensible to use a marker of coagulation to detect them. To analyse AnV+ MP, a calcium-containing buffer is required, so it is best not to assess these MP in PPP, but in suspensions, because of the risk of clotting.

3.4.5 Beads for sizing and enumerating

It is recommended that standard concentration of beads be used to assist with the enumeration of MP when doing flow cytometry [216, 244, 268]. In this study, there were 4 bead sizes used, with the lowest sized beads unable to resolve well on either side or forward scatter. It is possible that the lack of granularity and the reflective nature of the particles contribute to this. I was able to resolve 0.75μm and 1.1μm beads in both side and forward scatter, and was able to identify MP binding AnV and less than 1.1μm in size, but did not use the 0.3μm and 0.75μm beads to measure the MP sizes.

4 CD14+, CD66b+ and TF+ Microparticles

4.1 Background

Behçet's syndrome (BS) is a multisystem inflammatory disorder in which venous thrombosis is a common complication, occurring in 10-40% of cases and representing a leading cause of morbidity and mortality in the condition [450, 452, 453, 518]. A number of studies have investigated disorders of the coagulation cascade and fibrinolysis in BS [443, 559, 589], but none of their findings have translated into clinical practice [396, 524, 590, 591].

The last few years has seen increased appreciation of the importance of MP to disease [592], and the suggestion that tissue-factor expressing MP have a role in coagulation and clot propagation [593-597]. Moreover, numbers of circulating TF+MP have been related to clinical thrombosis [174, 212, 286, 315, 318, 597].

There is a clear need for biomarkers in BS. This will assist not only with development of new and alternative therapies, but also help to identify and improve risk management for patients, particularly with regards to thrombosis. In this chapter, we aimed to show that plasma cell membrane-derived MPs can be used to identify BS patients at risk of thrombosis.

4.2 Methods

4.2.1 Patients

Patients were identified and recruited as described in section 2.2.

4.2.2 Sample collection and preparation

Blood was collected as per section 2.4, and prepared as PPP as described in section 2.5. Microparticle preparation was performed as per section 2.6.

4.2.3 Annexin V, monoclonal antibody and microparticle sample preparation for flow cytometry

AnV and mAbs were added as per a 96-well plate plan using the concentrations calculated from the titration experiments documented in Chapter 3. The final concentrations and volume added of AnV and mAbs are documented in Table 3-1.

4.2.4 Flow cytometry

Flow cytometry was performed as described in sections 2.8 and 2.9.

4.3 Results

4.3.1 Patient demographics

Table 5-1 shows the patient demographics of the recruited population. This was a well-matched cohort with no significant difference between BS and HC in age and gender mix. All patients had mucocutaenous manifestations, with all having oral ulceration. Within the BS cohort, 21 (24%) had a history of thrombosis, of which 20 were male. This included 16 lower limb deep vein thromboses, 11 retinal vein occlusions (8 branch retinal vein and 3 central retinal vein), 2 superior vena cava occlusions and 1 dural sinus thrombosis. 6 patients had had more than one thrombotic episode, including 3 patients with a history of pulmonary embolus, 2 of whom had a previous deep vein thrombosis and 1 a dural sinus thrombosis.

Table 4-1 Demographic characteristics of Behçet's syndrome patients (BS) and healthy controls (HC)

	HC		BS	
Cohort size	74		88	
Median age in years (range)	41	(18 - 75)	43	(17 - 74)
Male sex (%)	40	54%	47	53%
Clinical Characteristics				
Mucocutaneous	0		88	100%
Erythema nodosum			29	33%
Pathergy or poor wound healing			48	55%
Musculoskeletal - arthralgias	0		71	81%
Arthritis			30	34%
Vascular	0		22	25%
Aneurysm			7	8%
Thrombosis			21	24%
Ocular	0			
Posterior/panuveitis	0		39	44%
Gastrointestinal inflammation	0		10	11%
Neurological	0		18	20%
BSDAI*	N/A		2	
Current medications				
Colchicine	0		25	28%
Azathioprine	0		17	19%
Mycophenolate	0		13	15%
Anti-TNF Therapy	0		16	18%
Cyclosporine	0		3	3%
Methotrexate	0		9	10%
Steroids†	0		30 (8.75mg)	34%

*BSDAI = Behçet's Syndrome Disease Activity Index (Median)
†Median dose in brackets

81% had musculoskeletal pain, of which 30% had had arthritis, which was a clinician diagnosis. Arthritis in BS is non-erosive and palindromic in pattern [400], so often this diagnosis cannot be objectively corroborated. 7 patients (8%) had had arterial aneurysms and there were no cases of arterial thrombosis. An ophthalmologist confirmed history of uveitis was present in 44% (39), and this was either posterior or panuveitis. Gastrointestinal involvement included diarrhoea and occasional bloody motions. Two had confirmation of gastrointestinal BS on colonoscopy, whilst the rest (8) reported irritable bowel-like symptoms presumed to

be secondary to BS. Neurological involvement included headaches and 3 patients had vestibular problems. The Behçet's Syndrome Disease Activity Index (BSDAI) is calculated from the clinical manifestations of BS and is out of 12. The Median BSDAI was 2, which indicates low disease activity.

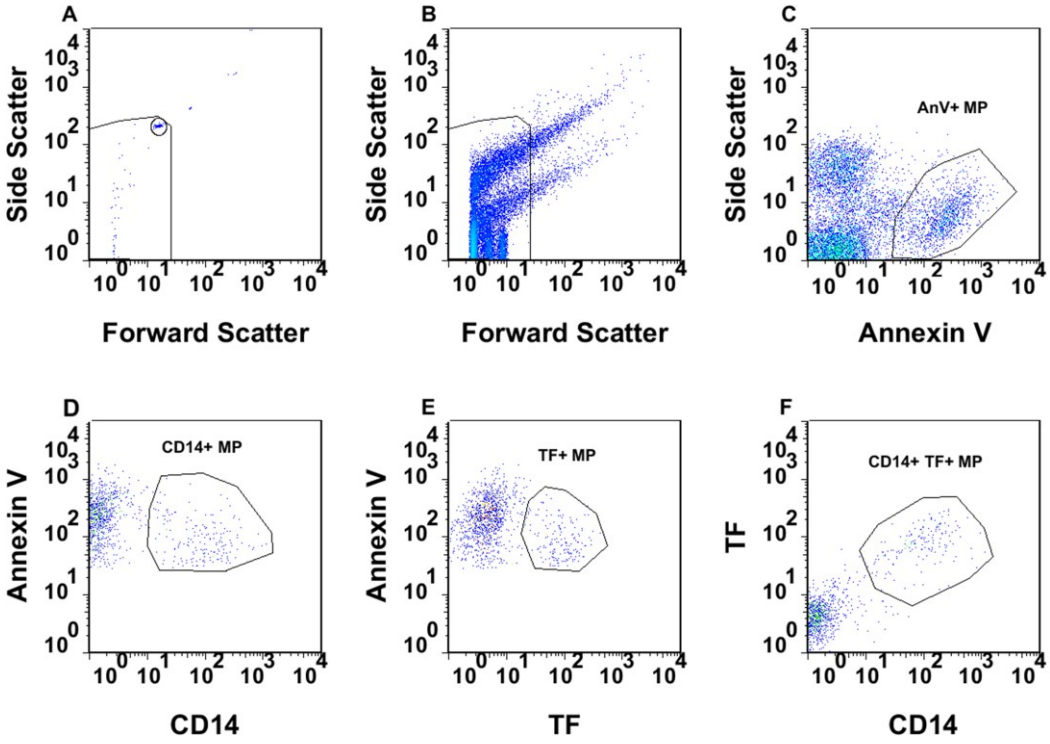
25 patients (28%) were on colchicine and 17 (19%) of patients took azathioprine, which was the most common immunomodulatory agent used. 16 (18%) took anti-TNF therapy, of which 12 were on adalimumab, with two each on infliximab and etanercept. 13 (15%) took mycophenolate, which was the second line DMARD of preference in this cohort. 3 (3%) patients took cyclosporine, whilst 9 (11%) took methotrexate. 30 (34%) of patients were on prednisolone with the median dose being 8.75 mg.

4.3.2 Gating strategy

As described in section 2.9, MP were enumerated and defined by size (less than 1µm in diameter), binding to AnV and specific antibodies. Figure 4-1 shows the gating strategy utilised to identify MP, in this case a triple-stained population of AnV binding CD14+ TF+ MP. Accurate gating reduced the risk of erroneous results, with loose gating overestimating event counts, whilst tight gating can lead to underestimating of event counts. Gating was done with isotype controls, so that positive binding of AnV and antibody could be compared. The x- and y-axes are in a logarithmic scale and represent the light collected by the detectors. Figure 4-1 A shows the position of the 1.1µm beads in the forward *versus* side scatter plot, which delineates the upper limit of MP detection. Figure 4-1 B shows the position of the MP suspension on the forward *versus* side scatter plot, with the gating identifying particles that are less than 1.1µm in diameter. The particles within this gate were then analysed in the side scatter *versus* AnV plot (Figure 4-1 C). This shows a

separate population, and a gate was put round these, identifying AnV+ MP. This population was used in further analyses in Figures 4-4 D – F. Figures 4-4 D and E show CD14+ and TF+ MP. Figure 4-4 F shows the CD14+ TF+ MP. These raw event counts were converted into MP count/ml using the formula in section 2.9.2.

Figure 4-1 Gating strategy for microparticle detection by flow cytometry



All MP were gated < 1.1µm in size using microbeads. **(A,B)** Forward and side scatter of (A) 1.1 µm microbead suspension and (B) plasma M ; **(C)** particles < 1.1µm binding AnV (AnV+) were defined as MP; **(D,E)** gating of AnV+ MP positive for (D) CD14 or **(E)** TF; **(F)** AnV+ MP stained by both anti-CD14 and anti-TF.

4.3.3 Quantification of microparticles in Behçet's Syndrome compared with healthy controls

Using the gating strategy as described in section 4.3.2, samples from the BS and HC groups were run to enumerate total AnV+, CD14+ and CD66b+ MP counts and the proportions of CD14+ and CD66b+ MP, which was given as a percentage of total MP (Figure 4-2). Results are expressed in medians. In Figure 4-2 A, the BS

patient group had an increased total MP count compared to the HC group (6.18 x 10^5/ml *versus* 1.90 x 10^5/ml; $p < 0.001$). Co-staining with antibodies showed a greater number of CD14+ MP in BS patient samples, both in total count (1.17 x 10^5/ml *versus* 0.20 x 10^5/ml; $p < 0.001$; Figure 4-2 B) and in proportion (16.97% v 9.18%; $p < 0.001$; Figure 4-2 C). As shown in Figures 4-2 D and E, there was increased CD66b+ MP in BS compared with the HC group both in count (0.46 x 10^5/ml *versus* 0.12 x 10^5/ml; $p < 0.001$) and proportion (7.83% *versus* 4.61%; $p < 0.01$), respectively. The total of CD14+ and CD66b+ MP counts did not account for the total MP count.

4.3.4 Quantification of tissue factor bearing microparticles in Behçet's Syndrome compared with healthy controls

In Figure 4-3, MP samples from the BS patient and HC groups were co-stained with TF antibody. The median TF+ MP counts and proportions were increased in the BS patient group compared with the HC group. As shown in Figures 4-3 A and B, total TF+ MP (0.53 x 10^5/ml v 0.07 x 10^5/ml) and the proportion of TF+ MP expressed as a proportion of total MP (8.72% v 3.66%) was increased in the BS patients compared to the HC. Samples TF+ MP were co-stained with CD14 (Figures 4-3 C and D) and CD66b (Figures 4-3 E and F) antibodies, and showed an increase in MP expressing TF that were deduced to be of monocyte or neutrophil lineages. Triple-stained CD14+ MP were increased in count (0.35 x 10^5/ml v 0.03 x 10^5/ml; Figure 4-3 C) and proportion (5.25% v 1.22%; Figure 4-3 D) in the BS patients compared to the HC group. Similarly, triple-stained CD66b+ MP was also increased in count (Figure 4-3 E) and proportion (Figure 4-3 F) in BS patient compared to HC group. All results showed $p < 0.001$.

4.3.5 Sub-analysis of microparticles in Behçet's Syndrome patients according to a history of thrombosis

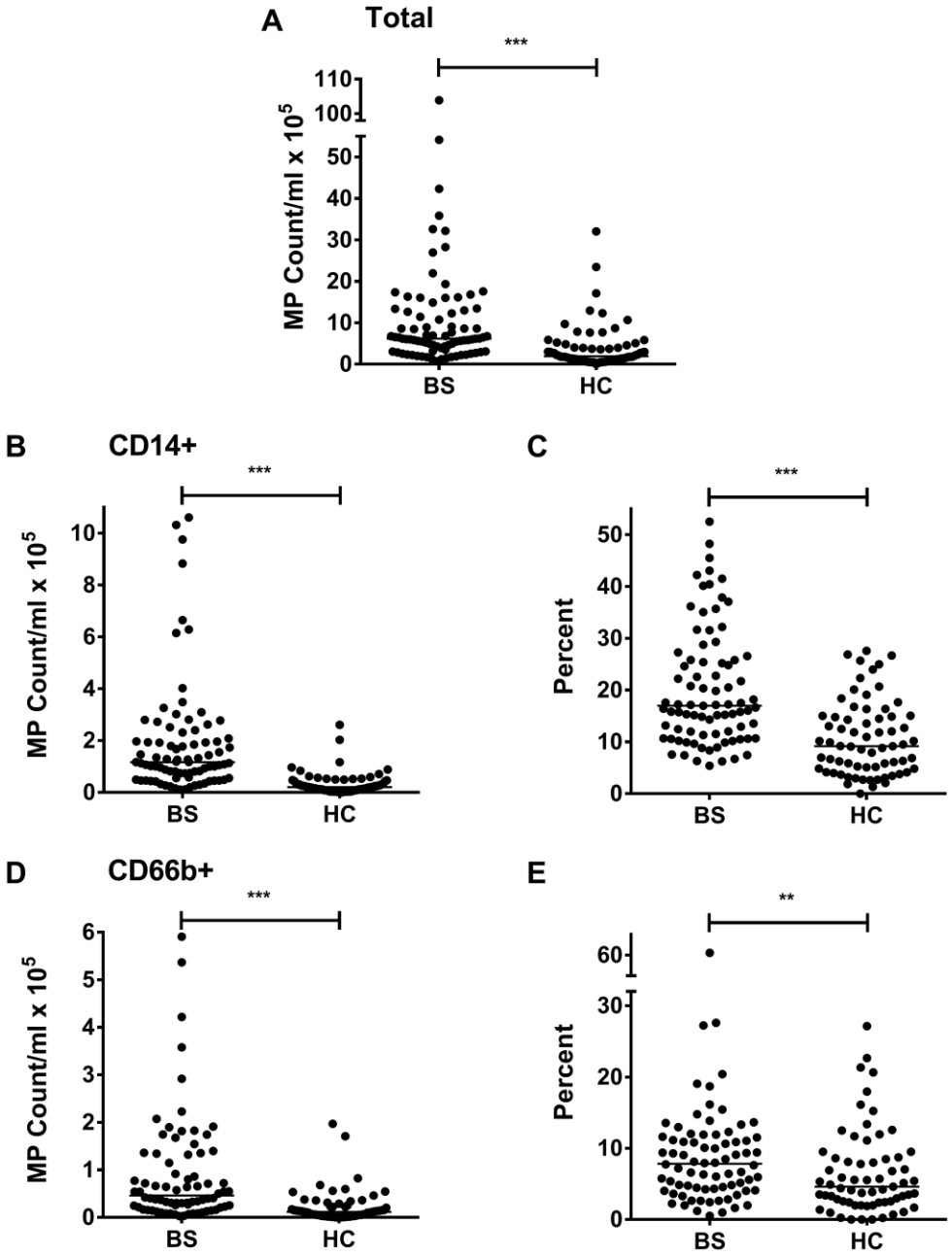
The BS patient group was split into those with (Th+) and without (Th-) a history of thrombosis. The median BSDAI in both groups was 2, with the maximum score being 12, so there was low disease activity in both groups (Figure 4-4) [571]. Overall, the median total, CD14+ and CD66b+ MP counts and proportions were increased in the Th+ than in Th- group, with proportions expressed as a percentage of total MP (Figure 4-5). As shown in Figure 4-5 A, the total MP count was increased in the Th+ compared with the Th- group (12.69 x 10^5/ml v 5.50 x 10^5/m; $p < 0.001$). This was the same for CD14+ (2.75 x 10^5/ml v 1.00 x 10^5/ml; $p < 0.001$; Figure 4-5 B) and CD66b+ MP counts (1.15 x 10^5/ml v 0.37 x 10^5/ml; $p < 0.01$; Figure 4-5 D). The proportion of CD14+ (23.70% v 15.75%; $p < 0.01$; Figure 4-5 C) and CD66b+ MP (9.40% v 5.60%; $p < 0.05$; Figure 4-5 E) were also higher in the Th+ than the Th- groups. This difference was not as great as that between BS patient and HC groups (Figure 4-2).

4.3.6 Sub-analysis of tissue factor bearing microparticles in Behçet's Syndrome according to a history of thrombosis

Figure 4-3 showed increased TF-expressing MP in the BS patients compared to the HC group. The BS patient group was sub-analysed to investigate median TF-expressing MP counts and proportions in Th+ *versus* Th- groups (Figure 4-6). Dual staining of MP with TF antibody showed increased count (1.84 x 10^5/ml v 0.42 x 10^5/ml; $p < 0.001$; Figure 4-6 A) and proportion (13.67% v 7.88%; $p < 0.001$; Figure 4-6 B) of total TF+ MP in the Th+ than Th- group. TF-expressing MP co-stained with CD14 antibody were increased in count (0.94 x 10^5/ml v 0.26 x 10^5/ml; $p < 0.001$;

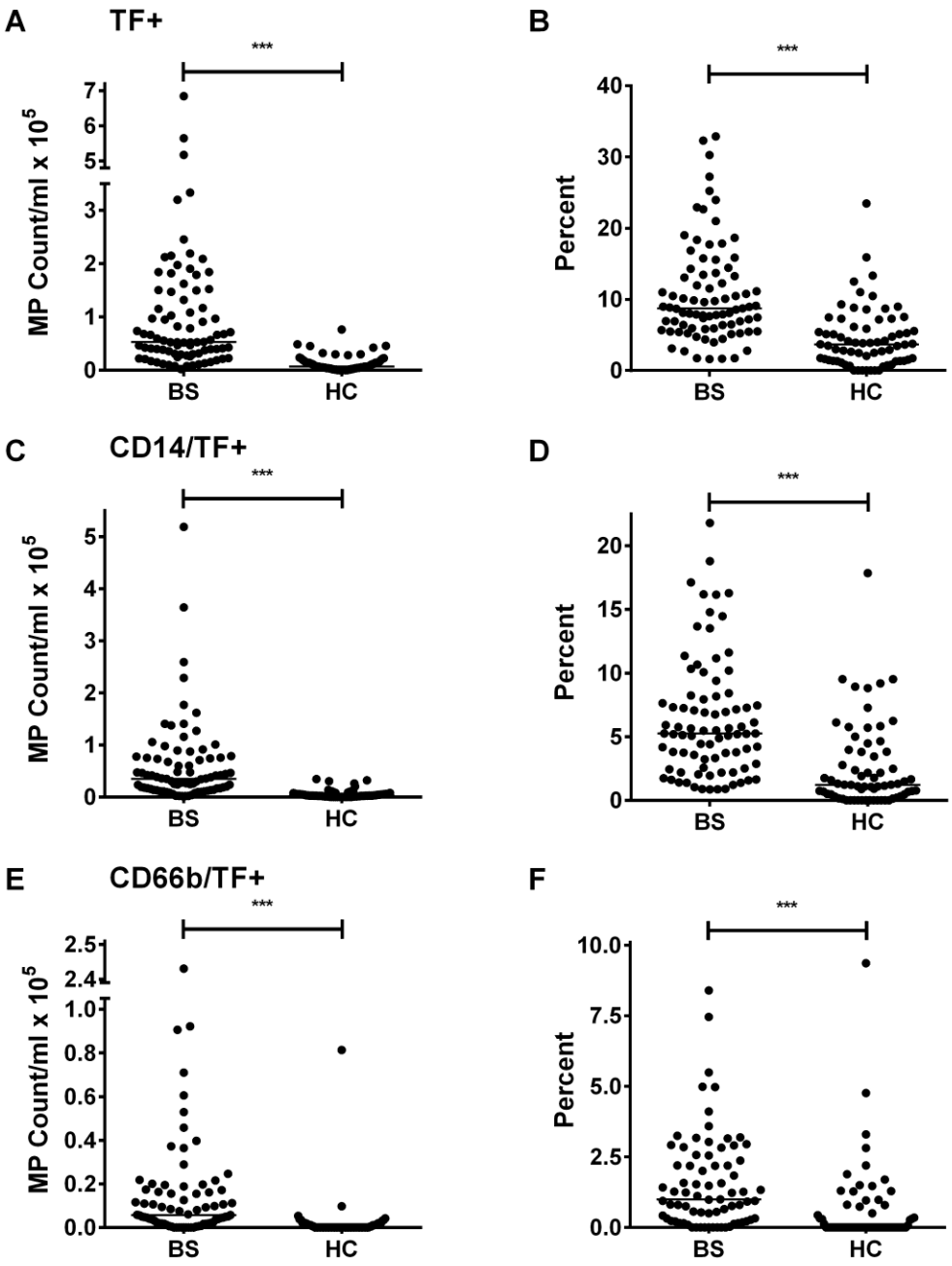
Figure 4-6 C) and proportion of CD14+ MP (7.47% v 5.13%; $p < 0.01$; Figure 4-6 D). CD66b+ TF+ MP were also increased in count (0.22×10^5/ml v 0.04×10^5/ml; $p < 0.001$; Figure 4-6 E) and proportion of CD66b+ MP (2.19% v 0.57%; $p < 0.001$; Figure 4-6 F) in the Th+ compared to the Th- group.

Figure 4-2 Total, CD14+ and CD66b+ microparticles counts in Behçet's Syndrome patients compared with healthy controls



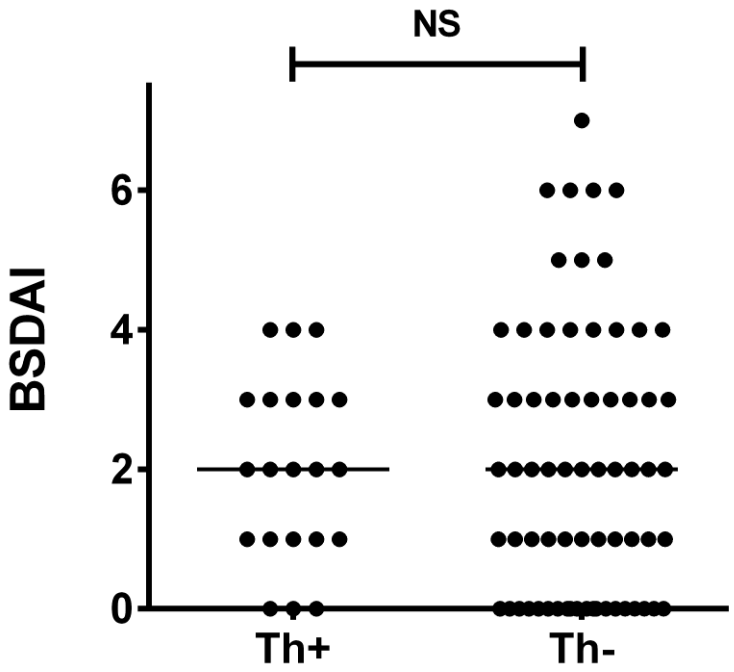
MP were enumerated from BS and HC samples by flow cytometry using AnV and monoclonal antibodies against CD14 and CD66b. **(A)** total MP; **(B,C)** CD14+ MP; **(D,E)** CD66b+ MP. Data are expressed as absolute counts **(A,B,D)** or % total MP **(C,E)**. **p < 0.01; ***p < 0.001.

Figure 4-3 Total tissue factor expressing microparticles and those that co-stain CD14 and CD66b in Behçet's Syndrome patients and healthy controls



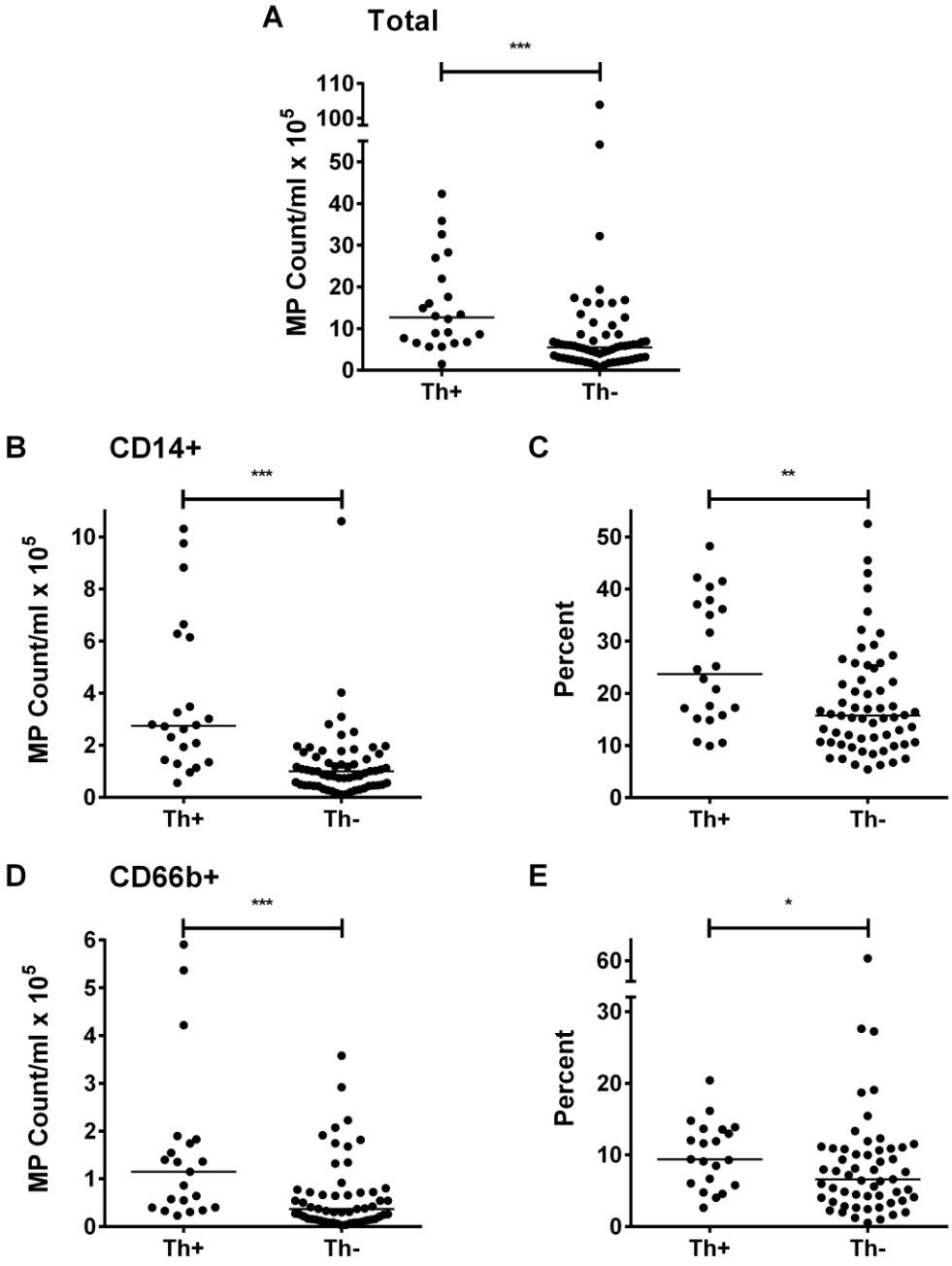
MP were enumerated from BS and HC samples by flow cytometry using AnV and monoclonal antibodies against CD14 and CD66b. **(A,B)** total TF+ MP; **(C,D)** CD14/TF+ MP; **(E,F)** CD66b/TF+ MP. Data are expressed as absolute counts **(A,C,E)** or % total TF+ MP **(B,D,F)**. ***p < 0.001.

Figure 4-4 Median Behçet's Syndrome Disease Activity Index (BSDAI) in Behçet's Syndrome patients with a history of thrombosis compared to those without



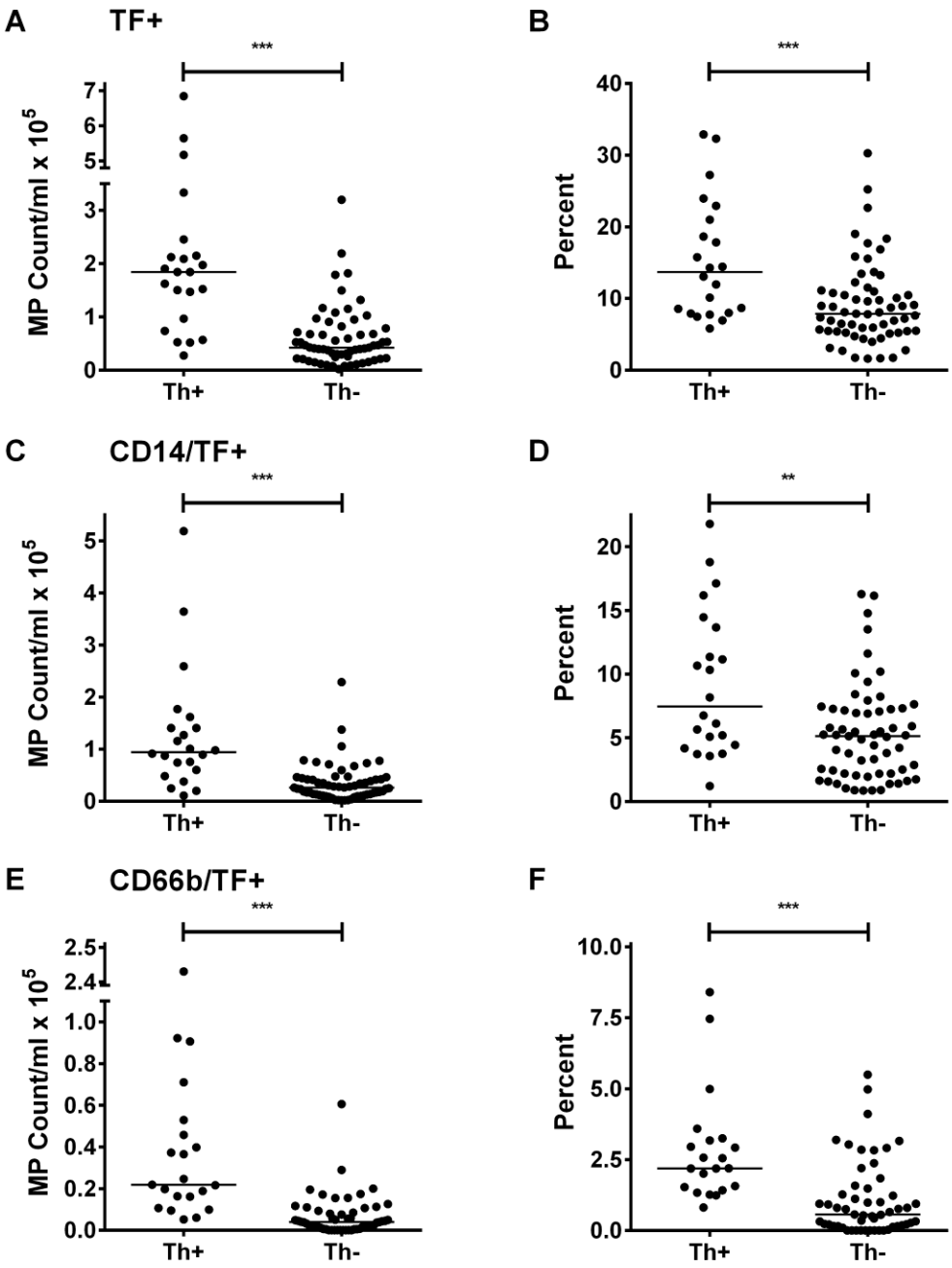
BSDAI of all patients participating was calculated on the day of recruitment and when the sample was taken. There was no significant difference in median BSDAI between BS patients with a history of thrombosis (Th+) and those without (Th-). The maximum score is 12, so the calculated median score in this group of patients represents a low disease activity.

Figure 4-5 Microparticles in Behçet's Syndrome patients with and without a history of thrombosis



MP were enumerated in Th+ *versus* Th- plasma by flow cytometry using AnV and mAb. **(A)** total MP; **(B,C)** CD14+ MP; **(D,E)** CD66b+ MP. Data are expressed as absolute counts **(A,B,D)** or % total MP **(C,E)**. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4-6 Tissue factor bearing microparticle In Behçet's Syndrome patients with or without a history of thrombosis



MP were enumerated in Th+ *versus* Th- plasma by flow cytometry using AnV and mAb. **(A,B)** total TF+ MP; **(C,D)** CD14/TF+ MP; **(E,F)** CD66b/TF+ MP. Data are expressed as absolute counts **(A,C,E)** or % total MP **(B,D,F)**. **p < 0.01, *** p < 0.001.

4.3.7 Variables and their impact on the results

Within the population of BS patients, there are several variables, which are known to have an impact on MP counts. Tobacco smoke induces MP generation from monocytes [598], circulating MP have been shown to be increased in women [599] and the procoagulation activity of plasma can increase with age [180].

The impact of smoking was reviewed by comparing non-smoking BS patients with HC (Figure 4-7) and a sub group analysis of BS patients, comparing those non-smoking Th+ with Th- (Figure 4-8). The median total (6.02 x 10^5/ml *versus* 1.90 x 10^5/ml; $p < 0.001$; Figure 4-7 A), CD14+ (1.13 x 10^5/ml *versus* 0.20 x 10^5/ml; $p < 0.001$; Figure 4-7 B) and TF+ MP (0.53 x 10^5/ml *versus* 0.07 x 10^5/ml; $p < 0.001$; Figure 4-7 C) were all increased in the non-smoking BS patient compared with HC group. Similarly, median total (10.69 x 10^5/ml *versus* 5.50 x 10^5/ml; $p < 0.01$; Figure 4-8 A), CD14+ (2.20 x 10^5/ml *versus* 1.00 x 10^5/ml; $p < 0.001$; Figure 4-8 B) and TF+ MP (1.50 x 10^5/ml *versus* 0.42 x 10^5/ml; $p < 0.001$; Figure 4-8 C) MP counts were all increased in Th+ non-smokers Th-.

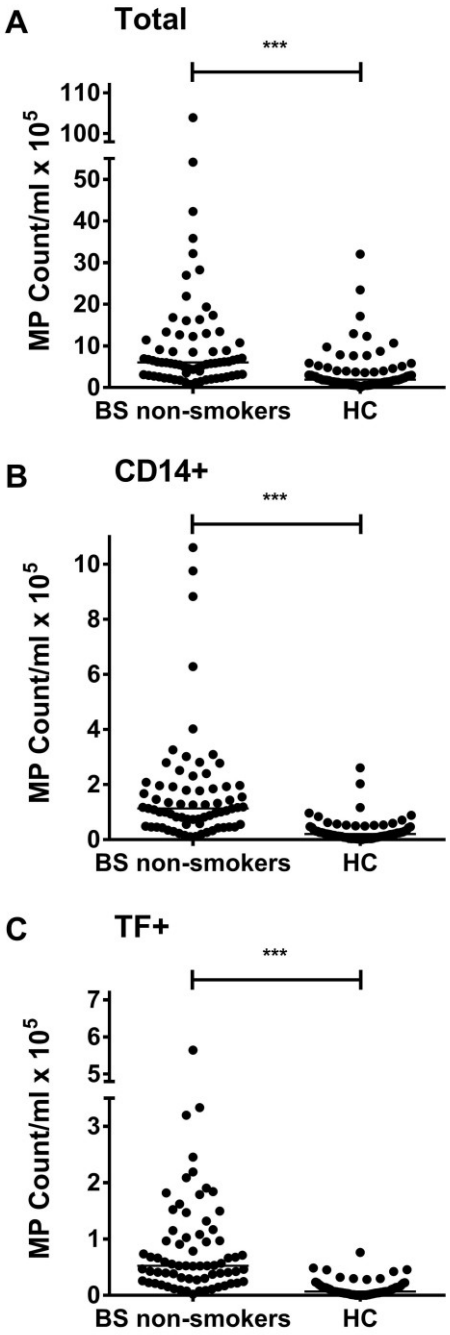
Gender comparisons were made of total, CD14+, TF+ and CD14+ TF+ MP (Figure 4-9). There was no difference in total MP count between male and female BS patients (7.31 x 10^5/ml *versus* 5.82 x 10^5/ml; Figure 4-9 A). The CD14+ MP (1.70 x 10^5/ml *versus* 1.00 x 10^5/ml; $p < 0.01$; Figure 4-9 B), TF+ MP (0.76 x 10^5/ml *versus* 0.42 x 10^5/ml; $p < 0.0.5$; Figure 4-9 C) and CD14+ TF+ MP (0.43 x 10^5/ml *versus* 0.28 x 10^5/ml; $p < 0.05$; Figure 4-9 D) MP counts were increased in males compared with females.

This increased MP count in males may have been explained by the increased frequency of thrombosis in male BS patients. In this cohort, 20 out of 21 patients with a history of thrombosis were male, and it is known that there is an increased frequency of thrombosis in male compared with female BS patients [429, 447, 450].

Therefore, I did a comparison of MP counts between male and female BS patients without a history of thrombosis (Figure 4-10). There was no difference in total (5.48 x 10^5/ml *versus* 5.78 x 10^5/ml; Figure 4-10 A) and CD14+ (1.09 x 10^5/ml *versus* 0.88 x 10^5/ml; Figure 4-10 B) MP counts in males compared with females respectively. Similarly, there was also no difference between TF+ (0.48 x 10^5/ml *versus* 0.41 x 10^5/ml; Figure 4-10 C) and CD14+ TF+ (0.24 x 10^5/ml *versus* 0.28 x 10^5/ml; Figure 4-10 D) MP counts in males compared with females, suggesting that the difference, albeit small, in MP counts between male and female BS patients was accounted for by Th+ patients.

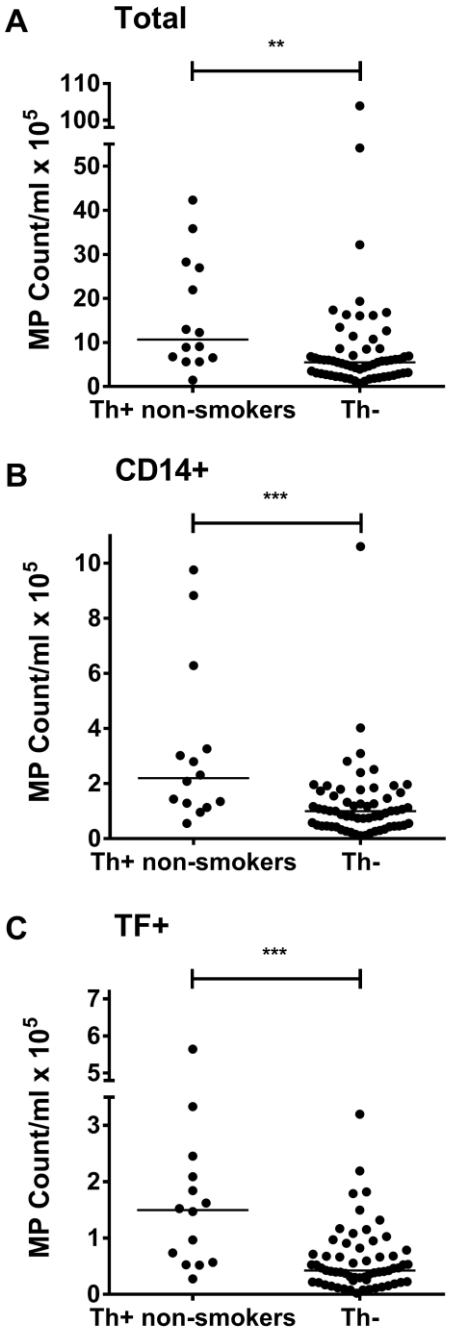
A comparison in BS patients of age with MP counts was also made (Figure 4-11). Figures 4-11 A and B show this comparison between age and MP counts with total and CD14+ MPs, whilst Figures 4-11 C and D show the TF+ and CD14+ TF+ subsets. There was no significant correlation found in any of the comparisons. BS patients were on various immunomodulatory therapies, so the numbers were too small to make a meaningful comparison, except for those on prednisolone (Figure 4-12) Comparisons were made between BS patients taking prednisolone and those without. There was no difference in total (5.99 x 10^5/ml *versus* 6.53 x 10^5/ml; Figure 4-12 A), CD14+ (1.02 x 10^5/ml *versus* 1.28 x 10^5/ml; Figure 4-12 B) TF+ (0.52 x 10^5/ml *versus* 0.63 x 10^5/ml; Figure 4-12 C) MP counts between those taking prednisolone and those who were not.

Figure 4-7 Non-smoking Behçet's Syndrome patients compared with healthy controls



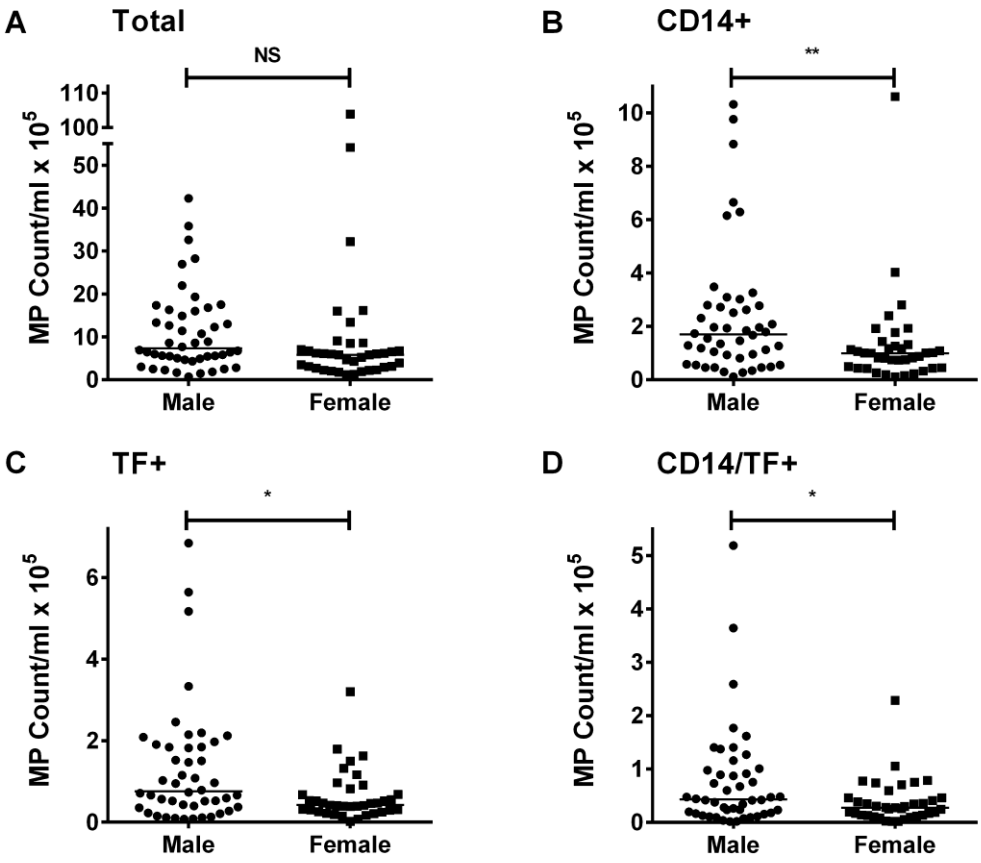
Non-smoking Behçet's Syndrome patients (BS non-smokers) were compared with healthy controls (HC). BS non-smokers have a higher total **(A)**, CD14+ **(B)** and TF+ **(C)** MP counts than HC. MP were enumerated in BS non-smokers *versus* HC samples by flow cytometry using AnV and mAb against CD14 and TF. *** $p < 0.001$.

Figure 4-8 Non-smoking Behçet's Syndrome patients with and without a history of thrombosis



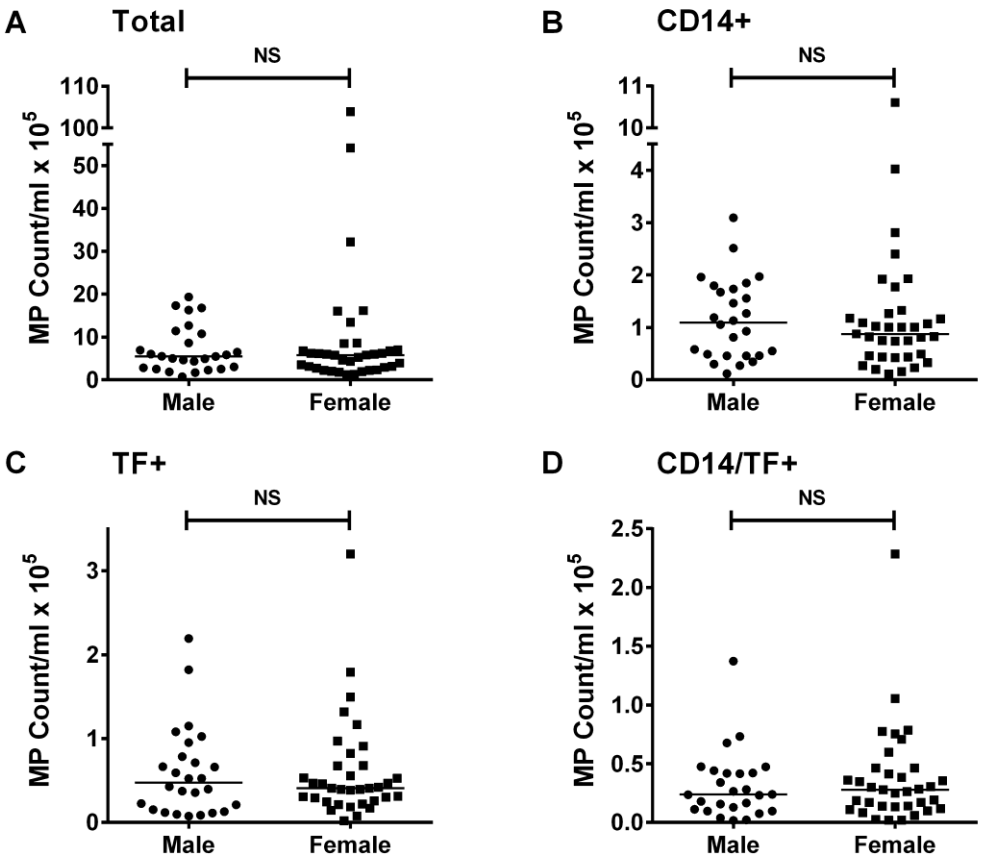
Non-smoking Behçet's Syndrome patients with a history of thrombosis (Th+ non-smokers) were compared with Behçet's Syndrome patients without thrombosis (Th-). Th+ non-smokers have a higher total **(A)**, CD14+ **(B)** and TF+ **(C)** MP counts than Th-. MP were enumerated in Th+ non-smokers *versus* Th- samples by flow cytometry using AnV and mAb against CD14 and TF. ** $p < 0.01$, *** $p < 0.001$.

Figure 4-9 Comparison of microparticle counts in male and female Behçet's Syndrome patients



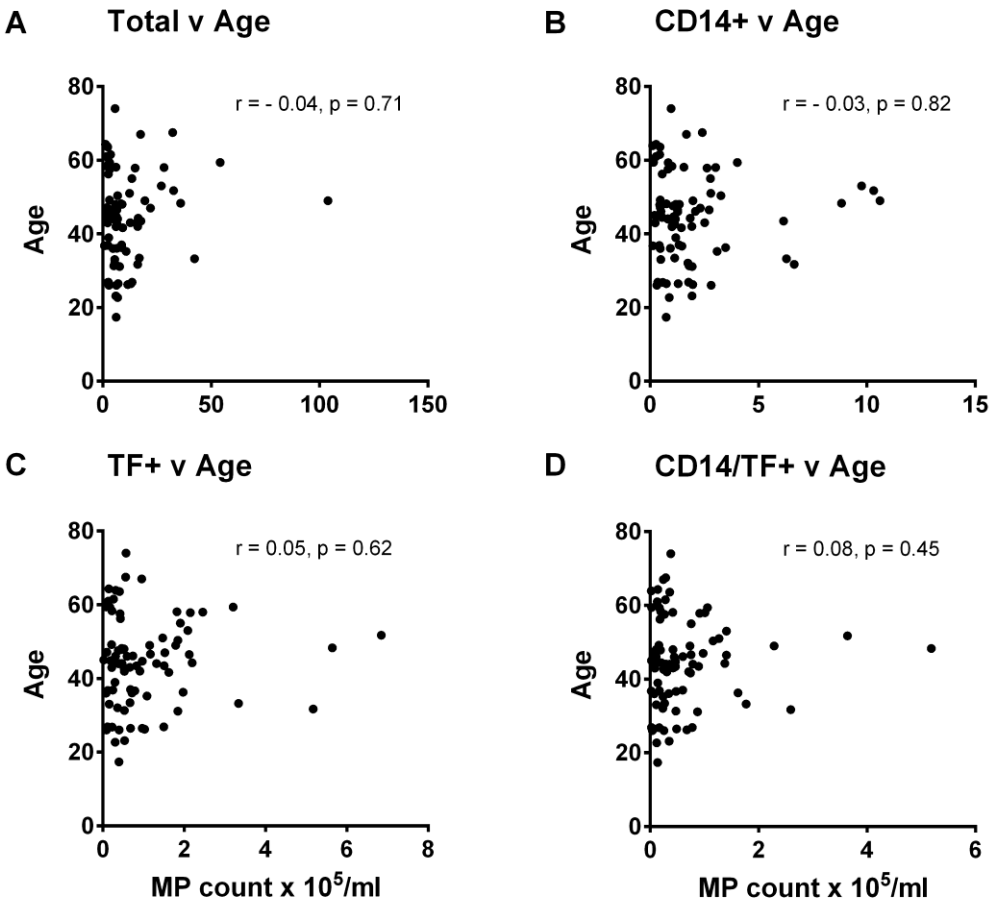
MP were enumerated in male and female BS patients. There was no difference in total MP counts between male and female BS patients **(A)**. There were higher CD14+ **(B)**, TF+ **(C)** and CD14+ TF+ **(D)** MP counts in male than in female BS patients. BS samples were assessed by flow cytometry using AnV and mAb to CD14 and TF. *p < 0.05, **p < 0.01.

Figure 4-10 Comparison of microparticle counts in male and female Behçet's Syndrome patients without a history of thrombosis



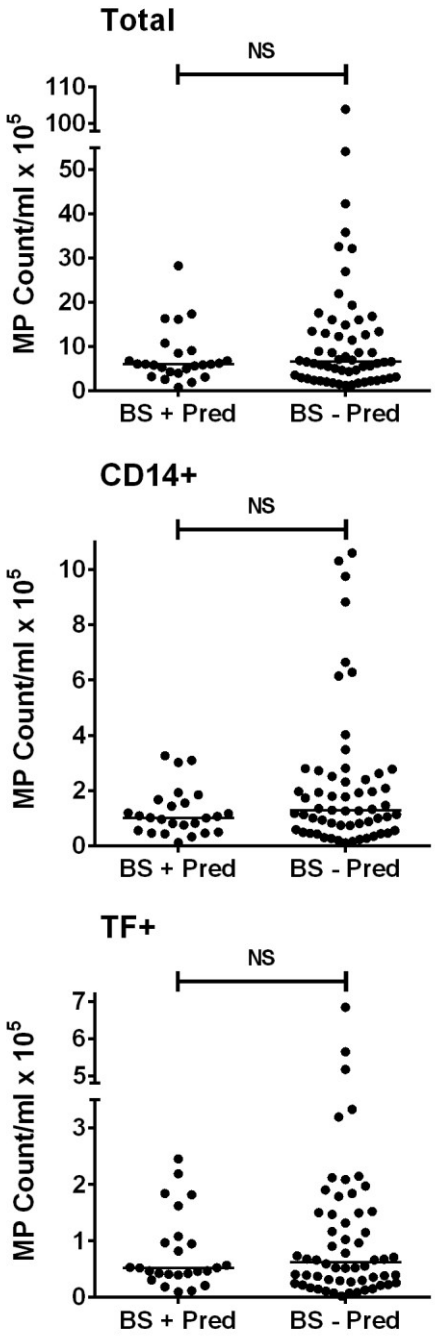
MP were enumerated in male and female BS patients without a history of thrombosis, the Th- patients. There was no difference in total MP counts **(A),** CD14+ **(B)**, TF+ **(C)** and CD14+ TF+ **(D)** MP counts between male and female Th- patients. BS samples were assessed by flow cytometry using AnV and mAb to CD14 and TF. NS = non-significant.

Figure 4-11 Correlation between age and microparticle counts in Behçet' Syndrome patients



Total **(A)**, CD14+ **(B)**, TF+ **(C)** and CD14+ TF+ **(D)** MP counts in BS patients compared with age. There was no statistically significant correlation found.

Figure 4-12 Comparison of total, CD14+ and TF+ microparticle counts in Behçet's Syndrome patients taking prednisolone compared to those without



MP counts were compared between BS patients taking prednisolone (BS + Pred) and those who were not (BS – Pred). Total **(A)**, CD14+ **(B)** and TF+ **(C)** MP counts were no different between the two groups. MP were enumerated by flow cytometry using AnV and mAb against CD14 and TF. NS = non-significant.

4.4 Discussion

4.4.1 Behçet's Syndrome population

While much of the BS population is as expected, an increased frequency of pulmonary embolism was observed in this cohort, which is higher than in much of the literature [366, 453, 518]. Pulmonary embolism frequency is thought to be low in BS, because the thrombi are strongly adherent to the inflamed endothelium, so it is recommended to treat patients with immunomodulatory therapy rather than anticoagulation alone [396, 565, 566].

Using immunomodulatory therapy only for the treatment of venous thrombosis in BS is more common in endemic than in non-endemic areas. This may be because of more rapid recognition of the underlying diagnosis in endemic areas. The three episodes of pulmonary embolism in this study cohort were diagnosed elsewhere prior to the patients being identified as having BS. Previous review of vascular complications in BS patients from 1984 – 2009 from my supervisor's cohort showed 62 episodes of venous thrombosis, 55 (89%) of who had been anticoagulated, but did not receive immunomodulatory therapy prior to referral [600]. Out of these 62 patients, 17 (27%) had a history of pulmonary embolism, which is also higher than expected and higher than in this current study. It is possible that these were not pulmonary emboli, but were inflammatory thromboses within the pulmonary artery, which are known to occur in BS [567]. It can be difficult to distinguish these from pulmonary emboli on imaging [518]. The CT pulmonary angiography and ventilation-perfusion scans for the three patients in this study with reported pulmonary emboli were not available for reanalysis.

4.4.2 Microparticle counts are increased in Behçet's Syndrome patients compared to healthy controls, and in Behçet's Syndrome patients with a history of thrombosis compared to those without

I have shown that total AnV binding MP are increased in BS patients compared to HC, and in BS patients with a history of thrombosis compared to those without. CD14+ and CD66b+ MP subsets, released from monocytes and neutrophils respectively, are also increased in BS and thrombotic BS patients compared to healthy controls and non-thrombotic BS patients respectively. TF expressing MP, including CD14+ and CD66b+ subsets, are also increased in BS patients compared with HC, and in those with a history of thrombosis compared to those without.

The increased level of MP in the BS patients with a history of thrombosis is a novel finding. Previous studies have shown CD62P+ MP to be increased in BS patients compared with HC, whilst the procoagulant activity of MP was also increased in BS patients, but neither study associated this increase with a phenotype [347, 601]. In the first study, the BS population was not well-defined, whilst in the second it was said that they did not find 'differences according to the clinical manifestations of BS patients, including those patients with and without venous or arterial thrombosis'.

BS is a heterogenous condition, which does not lend itself easily to the development of activity indices or biomarkers, and this heterogeneity also makes it difficult to accurately define a BS population and directly compare cohorts. Previously, oral ulceration was the *sine qua non* for diagnosis, but newer International Conference on Behçet's Disease criteria propose that oral ulceration does not need to be present for diagnosis [388]. This increases the sensitivity, but reduces the specificity

for diagnosis, which will increase the numbers diagnosed with BS, but may also include other conditions that are not BS.

There remains a reliance on clinically diagnosing BS, with no biomarkers to identify patients and those at increased risk of increasing activity. For example, arthritis in BS is asymmetric and palindromic in pattern, rather than persistent, often affecting the larger joints. It is non-erosive, so an alternative diagnosis ought to be considered, such as a seronegative arthropathy, if erosions are present [602]. Therefore, the diagnosis of arthritis in BS remains clinical, and since it is not persistent may have only presented a few times to the clinician.

There have been numerous attempts to link serological factors and tests to thrombotic risk in BS, but there are no serological markers or coagulation measures that impact on the management of BS. Consequently, EULAR guidelines on BS management were developed around utilising clinical markers of increased activity, such as the presence of uveitis or increased frequency of oral ulceration to guide management [396]. In a recent phase II trial of Apremilast, a small molecule inhibitor of phosphodiesterase 4, showed a reduction of mucocutaneous ulceration, with clinical measures the only marker of activity [398]. Although this was retrospective study, it is interesting that BS patients, and particularly those with the thrombotic phenotype, had increased levels of MP. This would warrant further investigation in a prospective study, to see whether MP have risen because of the thrombotic episode or whether it is a marker of risk for developing thrombosis. It ought to be noted, however, that the time between thrombosis and participation in this study was greater than a year in this study, making increased MP numbers as a consequence of thrombosis less likely.

4.4.3 Autoimmune conditions and microparticles

Increased MP levels are found in many inflammatory conditions [603, 604]. In some cases, MP is thought to drive pathology, while in others, MP may be a possible biomarker. In rheumatoid arthritis, MP are increased in inflammatory synovial fluid, leading to increased fibrin deposition [335]. In SLE, there are divergent findings on the level of MP and SLE activity. Some studies find that MP rise with disease activity [284, 605], whilst another study showed an overall decline, but increased AnV- sub-micron particles [273]. The decline in MP in SLE may be the result of consumption since MP are known to directly trigger complement activation [261, 336] and carry surface IgG1, which also triggers complement activation [606]. The increase in AnV- MP described in the Nielsen study of MP in SLE, may have been lipoproteins and chylomicrons, since the participants were not fasting, and it is known these can be confused for MP [607].

Patients with rheumatoid arthritis are twice as likely to have venous thrombosis than the general population [169]. A study reported that 7.2% of SLE patient have thrombosis [608], with raised lupus anticoagulant and the presence of anti-β2 glycoprotein I antibodies, in particular, associated with an increased risk of thrombosis [609]. It is a limitation of the present study that there was no inflammatory cohort as a comparison group. This would be to see whether inflammation was a risk factor in thrombosis. BS is associated with venous thrombosis, more so than other vasculitides and inflammatory conditions [348]**.** Both are associated with an increased risk of cardiovascular events, which is not just a reflection of increased disease activity, but of lipid profile derangement, including raised LDL [610, 611] [612-614]. In our cohort, the disease activity was low. Inflammatory markers were not checked, since ESR and CRP are often only modestly elevated or there is a discrepancy between recurrent oral ulceration and

CRP. For that reason, measurement of CRP and ESR are not part of the guidelines on BS management [396]. It is notable, however, that the median disease activity in the BS patient study cohort was low at 2 out of 12, so patients were quiescent.

4.4.4 Thrombosis and Behçet's Syndrome

MP level and activity have been shown to be increased in patients with venous thromboembolism [181, 592, 615]. Total MP have been shown to be increased in patients with venous thromboembolism, and in thrombotic patients due to malignant or idiopathic causes [178, 179, 273, 286, 332].

These findings suggest that increased MP is associated with a generalised increased risk of thrombosis and is not peculiar to one condition. Currently, BS patients with thrombosis are recommended to commence immunomodulatory therapy, since there is little evidence of benefit for anticoagulation. A small prospective Korean study showed no difference in recurrence of venous thromboembolism in patients on immunomodulatory therapy compared to combined immunomodulation and anticoagulation, whilst anticoagulation alone increased the risk of recurrence [566]. This outcome was consistent with previous French and Turkish retrospective studies of larger cohorts [453, 567, 568]. In non-endemic regions, there is a delay in the diagnosis of BS and may patients receive anticoagulation only, rather than in combination with immunomodulatory therapy [600]. Being a retrospective study is a weakness, but the differences remained despite patient being on immunomodulatory therapy. Also, sub group analysis to take into account smoking and prednisolone used, showed no difference. There was difference between male and female BS patients, but that was shown to be due to the increased frequency of BS patients with thrombosis (Figure 4-10). There was no association between MP count and age in this cohort, which was similar to the

finding of CD62P+ MP levels in patients with BS in a previous study. In that study, CD62P+ MP were found to be significantly greater in matched 20 – 30 and 31 – 50 year old BS patients than healthy controls, but not in those over 50 [601]. The proportion of CD62P+ MP varied between the BS patients and healthy controls, but there was no significant increase with age across either group [601]. Following a single venous thromboembolism, however, MP counts have been shown not to increase with age, but the procoagulant activity of MP did increase with age [180]. This may suggest that there is an increased proportion of TF+ MP, in the elderly population that would explain this, but this also was not found in this study.

4.4.5 Tissue factor bearing microparticles

The increased level of TF+ MP is a significant finding and is consistent with findings in other inflammatory conditions and malignancy [179, 315, 332, 616]. Herein, TF+ MP were not just increased in BS patients compared with HC, but also in BS patients with a history of thrombosis compared to those without. This does not imply causality, but does suggest an association. Similarly, TF+ MP are increased in malignancy patients with a history of thrombosis, compared to those without, suggesting an association with increased thrombotic risk in that group of patients [315, 318, 320].

Based on co-staining of MP with antibodies against CD14 and CD66, it is likely that TF+ MP derive at least in part from cells of monocyte and neutrophil lineage, although other cell types may also contribute to the circulating TF+ MP pool. Neutrophils are activated in BS [346] and neutrophils express CD14 at low density and have been shown to express TF [211, 332, 617]. Thus, the increase in TF+ MP from monocytes and/or neutrophils may provide a link between inflammation and thrombosis in BS.

5 CD62P and PSGL-1 bearing microparticles

5.1 Background

In chapter 4, flow cytometry of BS samples showed an increased total, TF+, CD14+ and CD66b+ MP count compared with HC. These MP were also increased in thrombotic compared to non-thrombotic BS. These findings were present despite BS patients being on corticosteroids and immunomodulatory therapy, showing an association between increased MP, especially TF+ MP, and BS patients with a history of thrombosis. There are, however, other factors that contribute to clot formation and propagation.

P-selectin (CD62P) is stored within α granules and Weibel-Palade bodies of platelets and endothelial cells respectively [618], and is a cell adhesion molecule important in recruiting leucocytes to the site of inflammation [339]. Trauma and injury lead to mobilisation of CD62P to the surfaces of endothelial cells and platelets, so they are ready to recruit leucocytes to the site of injury [619, 620]. This suggests that CD62P provides a link between inflammation and thrombosis.

Given its role in recruitment of cells to the site of injury, CD62P has been mooted as a target for therapy. Inhibitors of CD62P have shown promise in decreasing thrombus weight in mouse and baboon models of venous thrombosis [621, 622]. Clopidogrel, a thienopyridine derivative that inhibits the binding of ADP to its receptor on platelets, has been shown to decrease CD62P expression and platelet-leucocyte conjugate formation [623]. Increasing dose of clopidogrel has been associated with reduced peri-procedural myocardial damage following percutaneous coronary intervention, and was associated with reduced CD62P levels [624]. It has been shown that CD62P+ MP are increased BS patients compared to healthy controls, but this was not associated with a phenotypic difference [601].

P-selectin glycoprotein ligand-1 (PSGL-1) has also been shown to be a link between inflammation and thrombosis. It is a ligand that allows tethering of selectin expressing cells, such as leucocytes and platelets to the endothelial surface, thereby promoting leucocyte trafficking [625]. Furthermore, it is also expressed on the surface of monocytes, which are activated in thrombosis and co-express TF, so TF-expressing monocytes are recruited into the thrombus by binding to activated platelets, via the interaction of PSGL-1 and CD62P [626].

The presence of CD62P and PSGL-1 have been shown to be imperative for the propagation of thrombus in baboon and mouse models, with an inhibitor to PSGL-1 reducing thrombus weight [321, 329]. Cancer cell derived MP bearing PSGL-1 from patients infused into a living mouse, showed increased thrombus formation *in vivo* and the thrombotic state could be abrogated by using an anti-CD62P antibody [322]. As PSGL-1 is located on the surface of monocyte-derived TF+ MP, this leads to accumulation of TF within the clot, such that TF-expressing MP are the only source of TF within the propagating clot [627]. PSGL-1 bearing monocytes have been shown to be increased in patients with acute myocardial infarction, with intravascular imaging by optical coherence tomography showing that increased levels of these monocytes were associated with plaque rupture and intracoronary thrombus [628].

It is possible that a combination of CD62P+ and PSGL-1+ MP contributes to the finding of the low frequency of embolization that occurs in BS [438, 446, 447]. Increasing thrombus weight and a denser fibrin network, tends to lead to thrombi in BS being less likely to break off and embolise. In this set of experiments, I investigated whether there were increased levels of CD62P+ and PSGL-1+ MP in BS patients compared with healthy controls and whether this was especially increased in BS patients with thrombosis.

5.2 Methods

5.2.1 Patients

Patients were identified and recruited as described in section 2.2.

5.2.2 Sample collection and preparation

Blood was collected as per section 2.4, and prepared as platelet poor plasma (PPP) as described in section 2.5. Microparticle preparation was performed as per section 2.6.

5.2.3 Antibodies and MP sample preparation for flow cytometry

Antibody and MP samples were prepared as described in section 4.2.3.

5.2.4 Flow cytometry

The flow cytometer was cleaned as per protocol using proprietary cleaners and molecular grade H_2O. 1µm and 3µm bead suspensions prepared as per methods section 2.9, to confirm sizing and enumerating MPs respectively.

Each sample was run with 3µm run at the start, middle and end of each series of samples pertaining to a patient. The initial sample run was used to standardise total MP counts (AnV+ MPs) for each patient sample, and all subsets of plasma cell membrane derived MPs. Comparisons were made between BS patients and healthy controls (HC) and between thrombotic BS (Th+) and non-thrombotic BS (Th-) patients.

5.2.5 Statistics

All results are presented as medians and analysed by Mann-Whitney U comparisons, as detailed in section 2.15.

5.3 Results

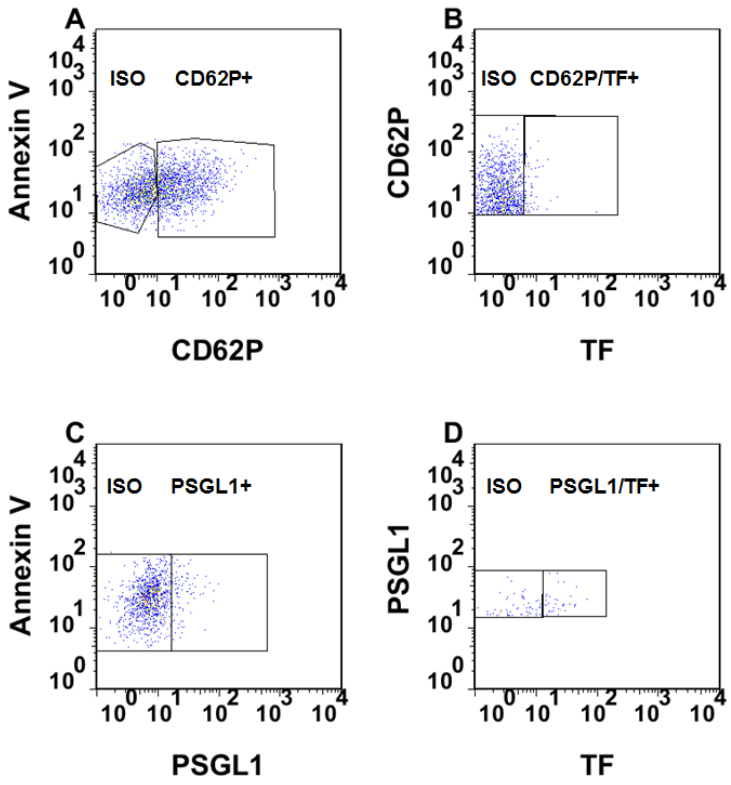
5.3.1 Patient demographics

Patient demographics and details are as previously described in section 4.3.1 and table 4-1.

5.3.2 Gating strategy used

As in section 2.9, all samples were enumerated and defined by size (less than 1μm in diameter), binding to annexin V and specific antibodies. Figures 4-1 A – C, shows how MP were gated for size, with the upper limit set using 1.1μm beads, and positive binding to annexin V. These MP less than 1.1μm in diameter positively binding to annexin V, were then incubated with monoclonal antibodies to PSGL-1, TF and CD62P (Figure 5-1). Figures 5-1 A and C, shows the gating strategy to identify CD62P and PSGL-1 bearing MP using isotype controls. Originally, gates were put around the isotype controls and any particles with increased fluorescence outside this gate was defined positive. Figures 5-1 B and D, show triple staining of TF+ MP with CD62P and PSGL-1 with gating set using isotype controls. Particles with increased expression outside the gate set for isotype controls were defined as being triple-stained and positively expressing TF on their surface.

Figure 5-1 Gating strategy CD62P+, PSGL-1+ and TF+ microparticle detection by flow cytometry



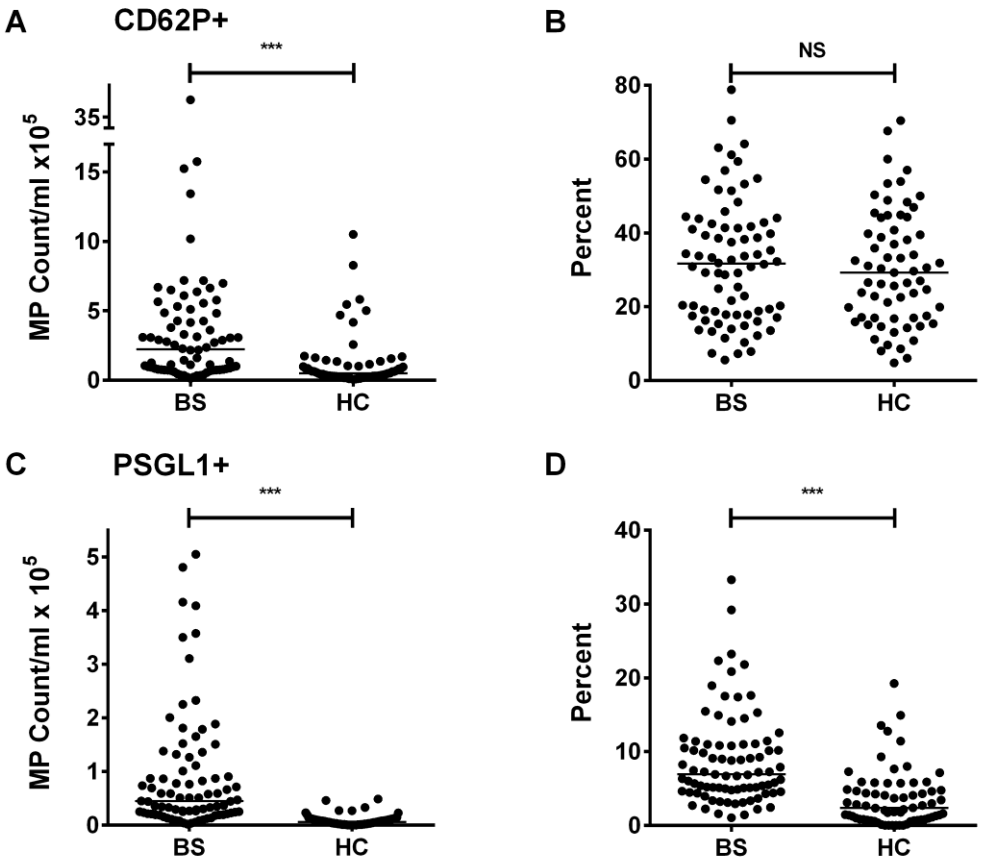
All MP were all positively identified as < 1.1µm in diameter and positively binding AnV as detailed in section 4.3.2. and Figure 4-1 A – C. CD62P+ MP **(A)**, CD62P/TF+ MP **(B)**, PSGL-1+ **(C)** and PSGL-1/TF+ **(D)** MP. ISO in **(A)** and **C** are PE-conjugated IgG1 and APC-conjugated IgG1 respectively. FITC-conjugated IgG1 in **(B)** and **(D)**. The ISO were used at the same concentration as their associated monoclonal antibodies to CD62P, PSGL-1 and TF respectively. ISO = isotype control antibody.

5.3.3 Quantification CD62P and PSGL-1 bearing microparticles in Behçet's Syndrome *versus* healthy controls

Using the gating strategy described in sections 4.3.2 and 5.3.2, samples from BS and HC groups were run to enumerate CD62P+ and PSGL-1+ MP counts and their proportions (Figure 5-2). The proportions are expressed as a percentage of total annexin V binding MP. The median counts of CD62P+ (2.23 x 10^5/ml *versus* 0.49 x 10^5/ml; $p < 0.001$; Figure 5-2 A) and PSGL-1+ (0.45 x 10^5/ml *versus* 0.06 x 10^5/ml; $p < 0.001$; Figure 5-2 C) MPs were increased in the BS patient group compared with the HC group. There was no difference in CD62P+ MP proportion (31.68% *versus*

29.27%; Figure 5-2 B) between the BS patient and HC group. The proportion of PSGL-1+ MP was increased in BS patients than HC (6.92% *versus* 2.37%; p < 0.001; Figure 5-2 D). The proportion CD62P+ MP was higher than PSGL-1+ MP, and higher than CD14+, CD66b+ and TF+ MP as shown in sections 4.3.3 and 4.3.4.

Figure 5-2 CD62P+ and PSGL-1+ microparticles in Behçet's Syndrome patients and healthy controls

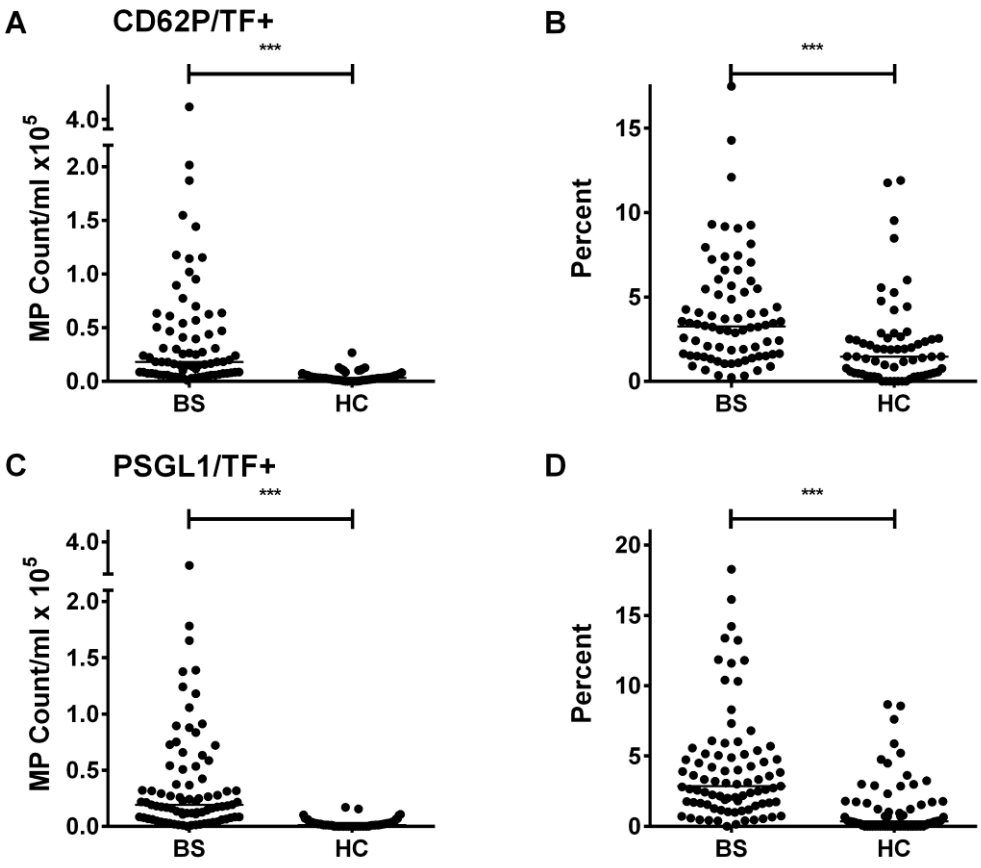


MP were enumerated in BS *versus* HC plasma by flow cytometry using AnV and mAb against CD62P and PSGL-1. **(A,B)** CD62P+ MP and **(C,D)** PSGL-1+ MP. Data are expressed as absolute counts **(A,C)** or % total MP **(B,D)**. ***p < 0.001; NS = non-significant

5.3.4 Microparticles bearing CD62P and PSGL-1, co-staining with tissue factor

In Figure 5-3, CD62P+ and PSGL-1+ MP were dual stained with a monoclonal antibody to TF. As described in section 4.3.4, median TF+ MP counts are increased in the BS patient group compared to the HC group. As shown in Figure 5-3 A and C, median counts of CD62P+ TF+ MP (0.18 x 10^5/ml *versus* 0.03 x 10^5/ml; $p < 0.001$) and PSGL-1+ TF+ MP (0.19 x 10^5/ml *versus* 0.01 x 10^5/ml; $p < 0.001$) were higher in BS patients than HC. The median proportion of CD62P+ TF+ (3.26% *versus* 1.46%; $p < 0.001$; Figure 5-3 B) and PSGL-1+ TF+ MP (2.86% *versus* 0.36%; $p < 0.001$; Figure 5-3 D) were also significantly higher in BS patients than HC. Median count and proportion of TF+ MP was also shown to be increased in BS patients than HC (section 4.3.4).

Figure 5-3 CD62P+ and PSGL-1+ microparticles co-stained with a monoclonal antibody to tissue factor in Behçet's Syndrome patients and healthy controls



MP were enumerated in BS and HC plasma by flow cytometry using AnV and monoclonal antibodies against CD62P, PSGL-1 and TF. **(A,B)** CD62P+ TF+ MP and **(C,D)** PSGL-1+ TF+ MP. Data are expressed as absolute counts **(A,C)** or % total MP **(B,D)**. ***p < 0.001.

5.3.5 Quantification of CD62P+ and PSGL-1 bearing microparticles in Behçet's Syndrome patients according to a history of thrombosis

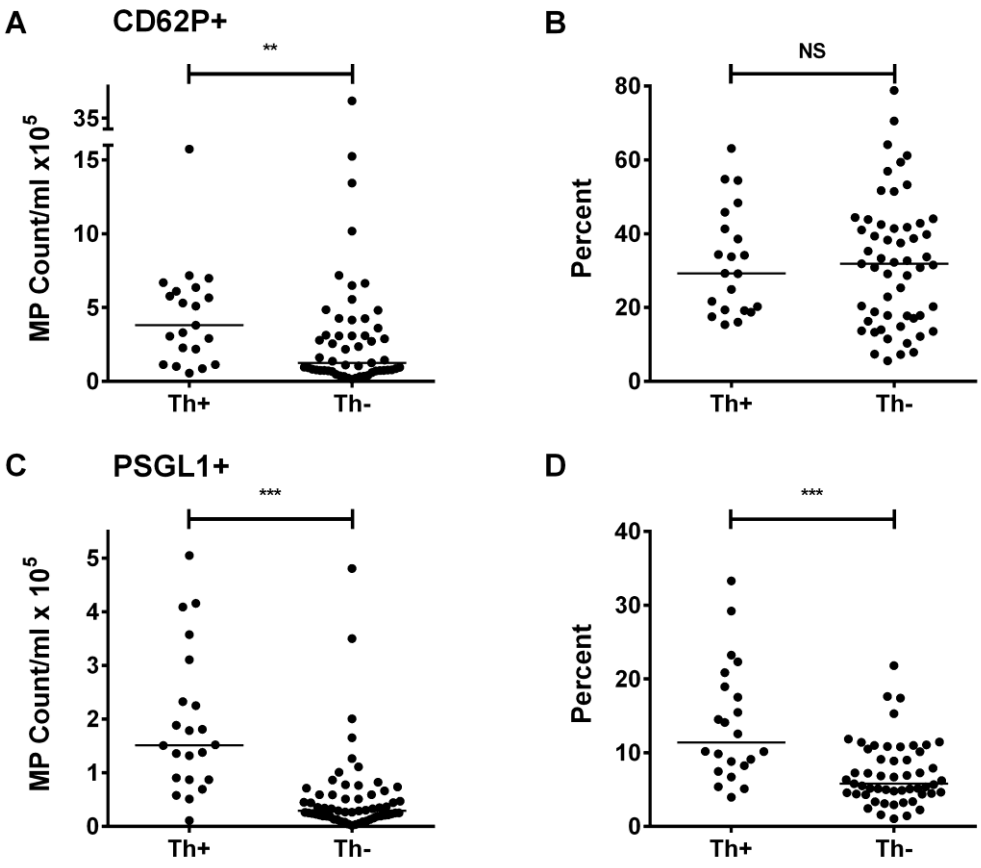
Further comparison of CD62P+ and PSGL-1 + MP median counts and proportions were seen in BS patients according to a history of thrombosis. In Figure 5-4, median count of CD62P+ (3.80 x 10^5/ml *versus* 1.26 x 10^5/ml; $p < 0.01$; Figure 5-4 A) and PSGL-1+ (1.51 x 10^5/ml *versus* 0.29 x 10^5/ml; $p < 0.001$; Figure 5-4 C) were

increased in Th+ compared with Th- patients. The difference in CD62P+ MP median count between Th+ and Th- patients, was not as great as the difference found between BS patients and HC (Figure 5-3 A). As in the BS patients *versus* HC comparison, there was no difference in the proportion of CD62P+ (29.24% *versus* 31.87%; Figure 5-4 B) between Th+ patients and Th-. The proportion of PSGL-1+ (11.36% *versus* 5.80%; $p < 0.001$; Figure 5-4 D) MP was increased in Th+ compared with Th-.

5.3.6 CD62P+ and PSGL-1 bearing microparticles co-staining with tissue factor in Behçet's Syndrome patients according to a history of thrombosis

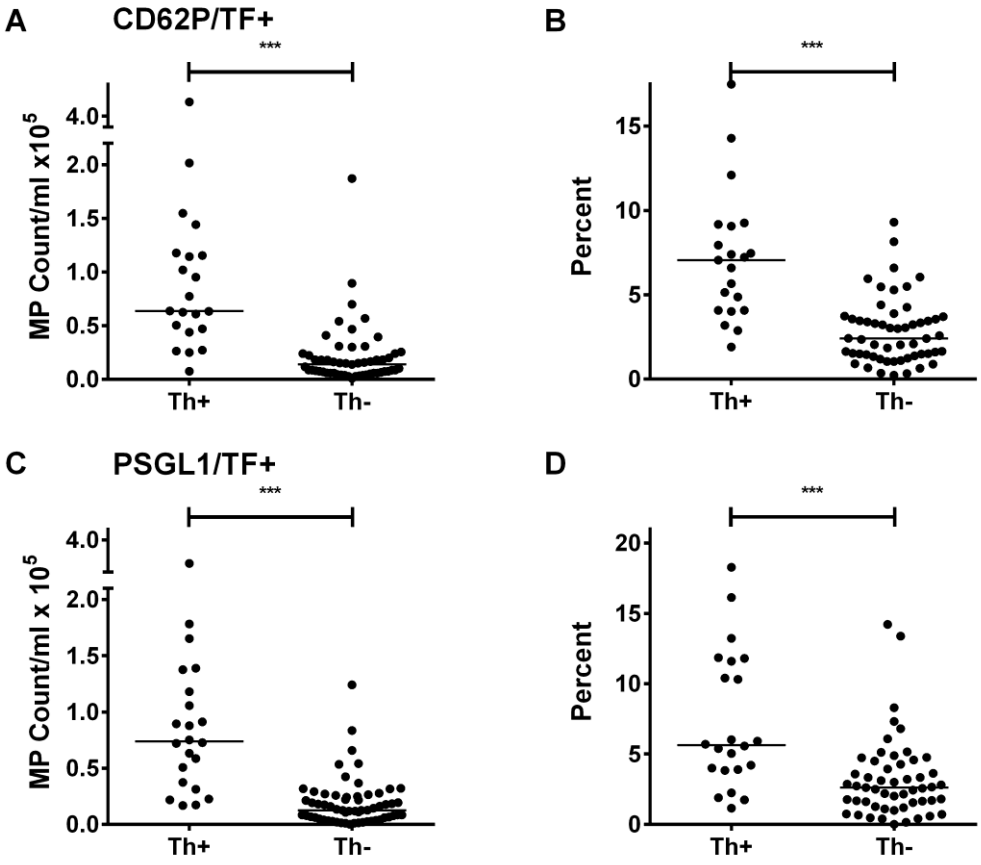
Figure 5-5 shows the comparison triple stained TF+ MP with either monoclonal antibodies to CD62P or PSGL-1 in Th+ or Th- patients. Median counts of CD62P+ TF+ (0.64×10^5/ml *versus* 0.14×10^5/ml; $p < 0.001$; Figure 5-5 A) and PSGL-1+ TF+ (0.74×10^5/ml *versus* 0.13×10^5/ml; $p < 0.001$; Figure 5-5 C) MP were increased in Th+ compared with Th- negative patients. The median proportions of CD62P+ TF+ (7.05% *versus* 2.40%; $p < 0.001$; Figure 5-5 B) and PSGL-1+ TF+ (5.64% *versus* 2.61%; $p < 0.001$; Figure 5-5 D) were also increased in Th+ compared with Th- patients.

Figure 5-4 CD62P+ and PSGL-1+ microparticles in Behçet's Syndrome patients with or without a history of thrombosis



MP were enumerated in Th+ and Th- plasma by flow cytometry using AnV and monoclonal antibodies against CD62P and PSGL-1. **(A,B)** CD62P+ MP and **(C,D)** PSGL-1+ MP. Data are expressed as absolute counts **(A,C)** or % total MP **(B,D)**. **p < 0.01, ***p < 0.001, NS = non-significant.

Figure 5-5 CD62P+ and PSGL-1+ microparticles co-stained with a monoclonal antibody to tissue factor in Behçet's Syndrome patients with and without a history of thrombosis



MP were enumerated in Th+ and Th- plasma by flow cytometry using AnV and monoclonal antibodies against CD62P, PSGL-1 and TF. **(A,B)** CD62P+ TF+ MP and **(C,D)** PSGL-1+ TF+ MP. Data are expressed as absolute counts **(A,C)** or % total MP **(B,D)**. ***p < 0.001

5.4 Discussion

5.4.1 CD62P-bearing microparticles are increased in count, but not proportion

Herein, I have shown that median count of CD62P+ MP is increased in BS patients compared to healthy controls and in Th+ compared to Th- patients. CD62P+ MP, however, as a proportion of total MP is not increased in BS patients compared to healthy controls, or in Th+ compared to Th- patients. It is expressed by endothelial

cells and activated platelets, and often constitutes a significant proportion of the circulating MP population in many studies [201, 334, 581, 629, 630]. This was also shown in this study. Since CD62P+ MP is released from two ubiquitous cellular sources, it is common that it makes up a substantial proportion of MP, so it is unlikely there is difference between disease and controls.

This result is similar to that found in the Royal London study, which showed an overall increase in CD62P+ MP in BS patients compared to healthy controls, but, as with the study demonstrating increased thrombogenicity of MP in BS, there was no correlation with a phenotype or clinical characteristic of BS [347, 601]. This link, as stated in chapter 4, remains the novel finding of this study.

The findings do not confirm a pathological role for CD62P+ MP in BS, nor it's thrombotic phenotype. It just shows an association of an increased count, but not an increased proportion. As mentioned earlier, antibody to CD62P in animal models led to a reduction in thrombus weight, which suggests a significant role for CD62P in thrombus propagation [621, 622]. Therefore, it is possible that CD62P+ MP is playing a significant role. Also, in view of the retrospective nature of this study, it is not clear whether the rise in CD62P follows the thrombotic event or precedes it. It seems unlikely that the rise in CD62P+ MP follows the episodes, since many of the patients presented a year after their embolic cause. It is likely that this is not specific to BS, but an upregulated mechanism of thrombosis. It would have been better to have inflammatory disease controls, but the disease activity in BS patients were low.

5.4.2 PSGL-1 bearing microparticles

This is one of the few studies to investigate PSGL-1+ MP. They have been shown to be expressed on monocyte-derived TF+ MP [321], accumulate within thrombus [322] and play an important role in thrombus propagation in a murine model of thrombosis [627]. Although it is possible that CD62P+ MP drop during an acute thrombosis [332], raised circulating level CD62P+ MP have been shown to associated with VTE [181]. Many of the PSGL-1+ MP are likely to co-express CD14, since they tend to be monocyte derived, and these tend to express TF [321]. Therefore, it makes sense there would be an increase PSGL-1+ TF+ MP when in Chapter 4 it was shown that CD14+ MP were increased.

5.4.3 Co-expression of tissue factor

TF co-expression was detected on both CD62P+ and PSGL-1+ MP. Most CD62P+ MP are likely platelet-derived, and platelets have not conclusively been shown to express TF. Non-stimulated monocyte-free platelets have been shown to express TF mRNA that is enhanced and induced by activation [631], TF pre-mRNA has been shown to be present in quiescent platelets [632], and spliced TF mRNA in platelets activated by sepsis [633]. Other studies, however, showed no TF activity from quiescent and ionophore stimulated platelets [325], and platelets free of monocytes do not express TF activity even after stimulation [634]. The latter group did how TF activity from platelets, following 2 hours of LPS stimulation of whole blood, but this was thought to be the result of the fusion of TF bearing MP with CD62P+ platelet-derived MP [635]. Moreover, leucocytes have been shown transfer TF to platelets via CD15 [636].

In vitro it is known that endothelial cells express TF, but it has also been shown to be in disease states and this leads to TF+ endothelial cell-derived MP. In sickle cell disease, there is an increase in TF-expressing endothelial cells and monocytes, which release TF-bearing MP promoting a procoagulant state that shortens the plasm clotting time compared with control MP [214]. It has also been shown that endothelial cells recycle TF-bearing MP to cell surface by endocytosis [215]. It is likely that the majority of CD62P+ MP come from activated platelets rather than endothelial cells, due to their ubiquity. From the literature, it remains controversial whether the platelet-derived CD62P+ MP express TF due to constitutive TF surface expression on platelets or as the result of aggregates with CD14+ MP. There is increasing use of Imagestream flow cytometry, which can take photographs of individual events and resolve down to 100nm [279]. This technique could show if there were aggregates of MP, and be able to detect dual- and triple-stained MP.

Furie's work has demonstrated not only that PSGL-1 played a significant role in thrombus propagation in a murine model of thrombosis, but that TF+ MP, particularly of monocyte derivation, are the only source of TF within the propagating thrombus [627]. It is known that PSGL-1 is expressed on monocytes, and it is likely that PSGL-1 positive expression is coming from monocyte-derived MP rather than any other source. This supports the model of thrombus propagation as described in Figure 1-4. Once the endothelium is injured, there is a release of cytokines, leading monocyte activation, release of CD14+ TF+ PSGL-1+ MP that accumulate within the thrombus due to binding of CD62P either of activated platelets or exposed endothelial cells, leading to their accumulation.

6 Functional assays

6.1 Background

The results from chapters 4 and 5 demonstrated that MP were increased in BS patients compared to healthy controls. Moreover, MP count and proportions were increased in BS patients with a history of thrombosis compared to those without thrombosis, in particular TF+ MP, which were increased both in count and proportion in the Th+ compared with Th- populations.

MP generate thrombin by a combination of TF-dependent and -independent pathways. The TF-dependent pathway, however, leads to more rapid thrombin generation and fibrin clots with denser networks that are resistant to fibrinolysis [262]. Studies by Mackman's group have demonstrated by thrombin, anti-thrombin and in house FXa generation assays that TF+ MP promote thrombin generation [178, 637]. This has been demonstrated in a variety of animal models, including murine models of venous thrombosis and a simian model of hypercholesterolaemia [305, 306].

MP have also been shown to generate thrombin in humans. MP from healthy individuals generate thrombin via a TF-independent pathway as measured by the generation of thrombin-antithrombin complexes [254]. In disease states, thrombin generation is increased in those with a history of thrombosis [301], in children with active vasculitis and thrombosis, [343] and SLE patients [284] than those without.

The purpose of this chapter was to show that MP from BS patients compared with healthy controls was associated with increased thrombotic risk by assessment of MP in coagulation assays. The hypothesis was that MP samples from BS patients, and particularly patients with a history of thrombosis, would generate more thrombin or FXa, as measured in the CAT and FXa generation assays respectively, and

would be helpful as an identifier of those at risk of thrombosis. Additionally, impaired fibrinolysis also occurs in BS, and raised plasminogen activator inhibitor 1 (PAI-1) and tissue activatable fibrinolysis inhibitor (TAFI) levels are thought to explain this [559, 638, 639]. There were three assays used: an in-house turbidimetric coagulation-lysis assay, the CAT generation assay and a proprietary FXa generation assay. Impaired fibrinolysis was assessed by the in the coagulation-lysis assay.

6.2 Methods and Materials

6.2.1 Sample collection

BS patients and healthy controls were recruited into the study as described in section 2.2 and peripheral blood samples were collected as described in section 2.4.

6.2.2 Preparation of platelet poor plasma

PPP was prepared following serial centrifugation of peripheral blood samples as described in section 2.5. PPP samples were stored at -80˚C in 500µl aliquots ready for analysis.

6.2.3 Preparation of microparticles and microparticle poor plasma

PPP was thawed in a 37˚C waterbath for 1 – 2 minutes, from which MP were prepared following an hour-long centrifugation, as described in section 2.6.

6.2.4 Coagulation lysis assay

The coagulation lysis assay is described in detail in section 2.12. The variation in this chapter was the use of CTI, FVII deficient plasma (Affinity Biologicals, cat no. FVII-DP) and an anti-TF neutralizing mAb (Sekisui Diagnostics cat no. 4509) to establish whether coagulation in this assay was a contact or TF-dependent process. As appropriate 6.5µg CTI and 0.5µg anti-TF neutralising antibody were used. The FVII deficient plasma was processed to produce FVII deficient MPPP as per section 2.6.

6.2.5 Calibrated automated thrombin generation assay

The CAT assay was performed as documented in section 2.13.

6.2.6 Zymuphen MP-TF assay

The Zymuphen MP-TF assay was used to assess MP-associated TF activity as described in section 2.14.

6.2.7 Statistics

Results were analysed as per section 2.15.

6.3 Results

6.3.1 Coagulation lysis assay

6.3.1.1 Tissue factor shortens the time to clot formation

I tested the coagulation step of the assay using normal pooled PPP, which was assayed five times. Figure 6-1 A shows the comparison between three curves that represent the extrinsic system, intrinsic system and without calcium. At each minute, there was a measure of turbidity using a spectrophotometer at absorption 405 nm. Each point of the curve represents the mean of five readings with the bars representing the standard error of the mean (SEM). In Figure 6-1 A there is no clot formation without the addition of 2.5µl 1M calcium and 6pM TF leads to a much shorter time to clot formation, also known as the clot lag time ($Tlag_c$) compared with no TF (intrinsic system). Figure 6-1 B showed shows the times for $Tlag_c$ stated as mean ± SEM. The $Tlag_c$ was much shorter for the extrinsic system (44s ± 4s) than the intrinsic system (14min 18s ± 1min 25s). Figure 6-1 C shows the $Tlag_c$ for PPP with increasing concentration of TF. As TF increases in concentration, there is a shortening of the $Tlag_c$ in a dose-dependent response.

6.3.1.2 Concentration of reteplase to be used in the coagulation lysis assay

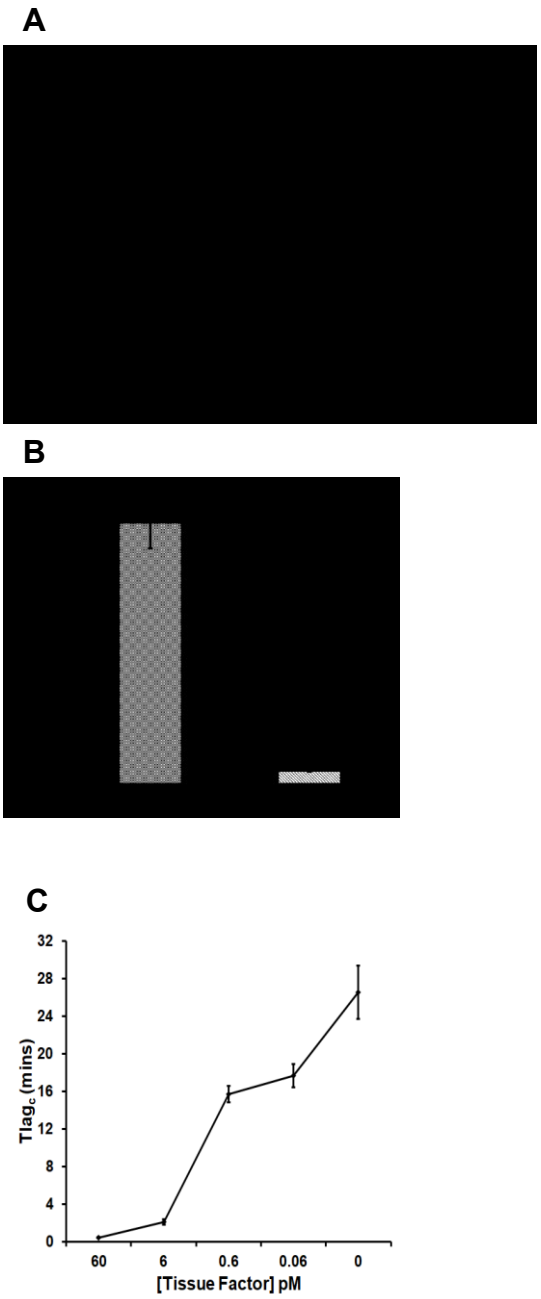
Following confirmation of the coagulation step of the assay, the lytic phase was assessed. The fibrinolytic agent used was reteplase, which is a fibrin specific serine protease and a third-generation form of tPa [578]. As described in section 2.12, reteplase was reconstituted from its powder using 10ml of water that was provided by the manufacturer, which gave 10U in 10ml volume and was stored in 50µl aliquots, which was the equivalent of 50mU. In the clinical situation, 10U is injected

and the circulating volume of blood in humans is approximately 5L. Therefore, the amount of reteplase per 100µl of blood is 0.2mU.

Thus, an experiment was set up to work out how much reteplase was required to induce lysis in this assay. Normal pooled PPP was run five times with varying amounts of reteplase. There was no exogenous TF added with only calcium was added to induce clotting. Turbidity readings were taken every minute for 60 minutes with a spectrophotometer set to read at absorption 405nm. Each point is a mean of five readings with the bars representing the SEM.

Figure 6-2 showed the results of this assay with 7 concentrations of reteplase from 0.01mU to 10mU/100µ/PPP assayed. At the lowest concentration of 0.01mU/100µl PPP there was no lysis induced. There was lysis induced with concentrations 0.05mU, 0.1mU and 0.5mU/100µl PPP. With increasing concentration of reteplase, there is a shift of the curve to the left, with shortening of the $Tlag_c$. Subsequently, at concentrations 1mU, 5mU and 10mU/100µl PPP, lysis is impaired with the curves moving further to the left, showing further shortening of the $Tlag_c$

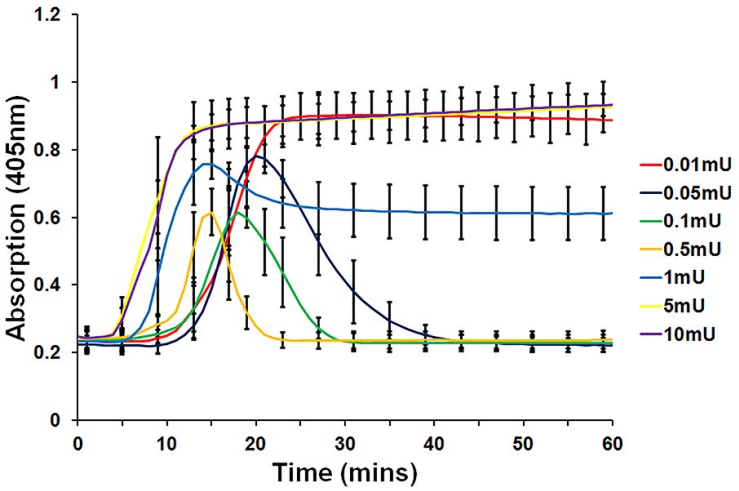
Figure 6-1 Turbidimetric coagulation assay to confirm the impact of calcium and increasing tissue factor concentration.



These graphs document the initial set-up of the assay and confirm the impact of calcium and TF. Each point represents the mean of samples from five healthy individuals, with each sample run in quadruplicate. The bars represent standard error of the mean. **(A)** shows the coagulation curves of the intrinsic system with the addition of 2.5µl 1M calcium, the extrinsic system with the addition of 1µl 6000 pM TF and without the addition of calcium. **(B)** shows a bar graph comparing the $Tlag_c$ of the intrinsic system with that of the extrinsic system. **(C)** shows that there is increasing $Tlag_c$ with declining concentration of TF.

In Figure 6-2, the intrinsic system was assessed, but further measures were taken assessing the extrinsic system and, therefore, the impact of TF (Figure 6-3). TF was added at a concentration of 6pM alongside reteplase, which was assessed at varying concentrations. The measures taken were $Tlag_c$, the time to lysis ($Tlag_l$), maximum rate of increasing turbidity (V_{MAX}) and the area under the curve (AUC). As before, PPP samples were run five times, with each point representing the mean and the bars representing the SEM. Figure 6-3 A shows declining $Tlag_c$ with the intrinsic system as reteplase concentration increased. There is little impact from reteplase on the $Tlag_c$ following the introduction of TF. The $Tlag_c$ seems to decline from concentrations 0.01mU – 0.1mU/100µl PPP, and then rises gently from concentrations 0.5 – 10mU/100µl PPP. At all concentrations, the $Tlag_c$ remains shorter for the extrinsic system than the intrinsic system.

Figure 6-2 Reteplase titration series (N = 5)



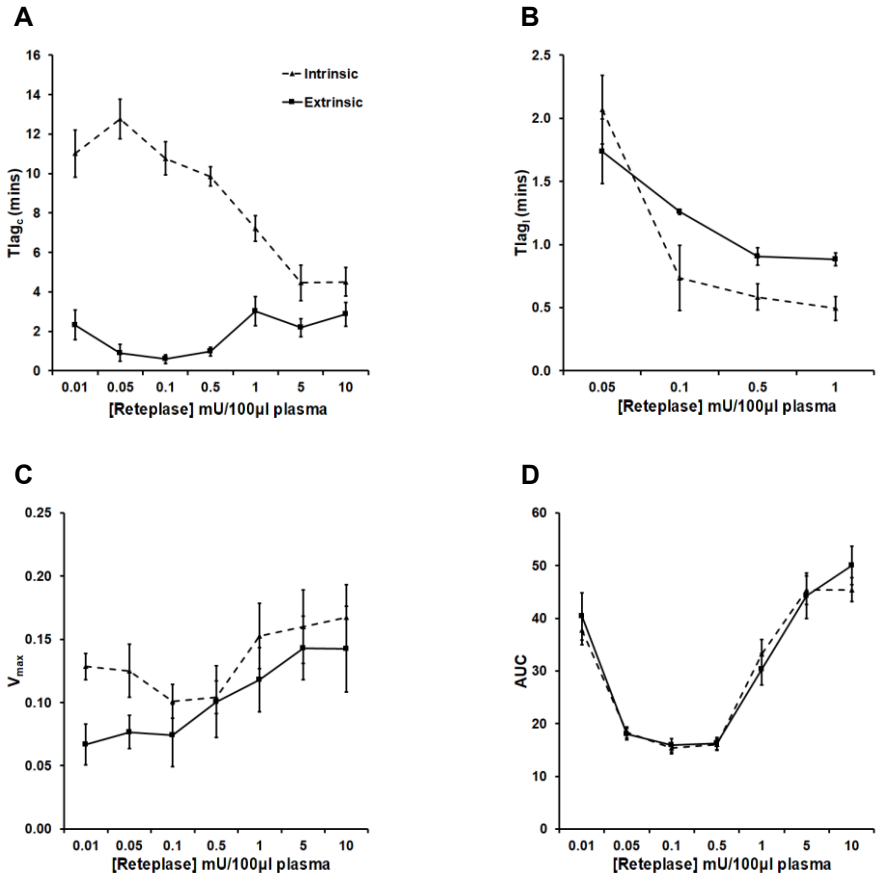
This assay was a reteplase titration series (n = 5) to identify the concentration of reteplase to use in the coagulation lysis assay. Each point on the curves represents the mean of five healthy individuals, with each sample run in quadruplicate, and the bars representing the SEM. As the concentration of reteplase increase, the curves shifted to the left, suggesting a shortening of the $Tlag_c$. Fibrinolysis occurs with reteplase concentration at 0.05mU/100µl PPP. No fibrinolysis was seen once reteplase concentration was greater than 1mU/100µl plasma.

In Figure 6-3 B, the impact of variable reteplase concentrations on lysis was assessed. The concentrations of reteplase assessed were from 0.05 – 1mU/100µl PPP, because these were the only concentrations that lysis was induced. This showed a shortening of the $Tlag_I$ with increasing concentration of reteplase to 1mU/100µl PPP, both in the intrinsic and extrinsic systems. The $Tlag_I$ tends to be shorter with the intrinsic than the extrinsic system.

Figure 6-3 C shows the V_{MAX} of increasing clot turbidity with varying concentration of reteplase in both the intrinsic and extrinsic systems. The V_{MAX} increases with increasing reteplase concentration in both systems. The V_{MAX} tends to be higher in the intrinsic rather than the extrinsic system. Figure 6-3 D shows the AUC with increasing reteplase concentration. The variation in the AUC is similar in both the intrinsic and extrinsic systems. The AUC drops with the induction of lysis, at its smallest at a reteplase concentration of 0.1mU/100µl PPP, and then increases with impairment of lysis with increasing concentration of reteplase.

Following these studies, the concentration of reteplase taken forward for use in the coagulation lysis assay was 0.1mU/100µl PPP. This showed coagulation and lysis phases, with less of an impact on $Tlag_c$ and $Tlag_I$ by reteplase at this concentration.

Figure 6-3 Coagulation-lysis assay comparing the impact of reteplase concentration on coagulation of platelet poor plasma (PPP) in the intrinsic and extrinsic systems

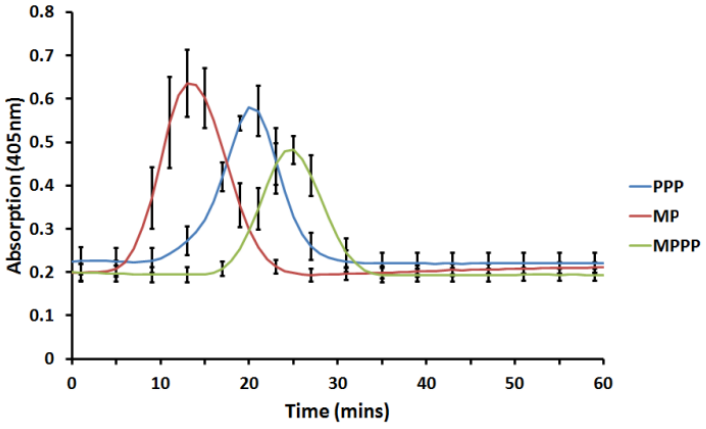


These graphs show the impact of increasing concentration of reteplase on both the intrinsic and extrinsic systems. The assay was run in PPP samples from 5 healthy individuals, with each sample run in quadruplicate. The bars represent standard error of the mean (SEM). The concentration of TF was 6pM. **(A)** shows the impact of reteplase on the lag time to clot formation ($Tlag_c$). **(B)** represents the time to lysis from peak thrombin generation ($Tlag_I$). **(C)** V_{MAX} represents the maximum rate of clot formation. **(D)** shows the AUC, which is the area under the curve. There was a separation of the intrinsic and extrinsic curves with the $Tlag_c$ and $Tlag_I$ measures **(A,B)**. There was crossover between the two curves in V_{MAX} and AUC measures at reteplase concentration 0.5µl/100µl and 0.05µl/100µl respectively **(C,D).**

6.3.1.3 Lag time to clot formation shortens with MP

After confirmation that the coagulation and lysis phases of the assay worked with PPP, the assay was run to check the impact of MP adhered to the well surface on coagulation and lysis phases of the assay. The MP were compared with the coagulation lysis profiles of autologous PPP and MPPP. MP were prepared as in section 2.6 in a 96-well plate. The MP wells were pre-incubated with 100µl MP suspension for 60 minutes at 37˚C. Following this step, the 100µl supernatant was removed and discarded, leaving the adhered MP on the well surface. The wells with autologous PPP and MPPP were pre-incubated with 100µl molecular grade water only, and this was removed and discarded as with the MP wells.

Figure 6-4 Coagulation lysis curves of microparticles compared with autologous platelet poor plasma (PPP) and microparticle poor plasma (MPPP) (n = 5)



These are the coagulation lysis curves of five healthy individuals, using a reteplase concentration of 1mU/100µl PPP. Each point represents the mean of five healthy individuals, and each sample was run in quadruplicate. The bars represent the SEM. These curves show that peak turbidity increases, with MP > PPP > MPPP. Also, $Tlag_c$ shortened, with MP < PPP < MPPP.

Figure 6-4 shows the coagulation lysis curves comparing MP, PPP and MPPP. The plasma used for the MP wells was autologous MPPP. The results are from five healthy individuals, with each healthy individual sample run in quadruplicate. Each point represents the mean of five individuals, with the bars representing SEM. There

was coagulation and lysis in all three, with a rise and decline in turbidity, as measured by the absorption at 405nm. The main finding was a shift to the left, representing shortening of the $Tlag_c$ with MP < PPP < MPPP.

Figure 6-5 shows the comparison of $Tlag_c$, $Tlag_l$, V_{MAX} and AUC of the MP, PPP and MPPP using bar graphs. V_{MAX} is the maximum velocity of increasing turbidity as the clot forms and AUC is the area under the curve. PPP were taken from five healthy individuals and each sample was run in quadruplicate. The results are presented as mean ± SEM. The $Tlag_c$ is shorter with MP than the PPP and MPPP (8min 6s ± 1 min 59s *versus* 14min ± 1min 30s *versus* 19min 23s ± 40s; Figure 6-5 A). There was a difference in $Tlag_c$ of MP compared to PPP ($p < 0.01$) and with MPPP ($p < 0.01$), but there was no difference between PPP and MPPP. Since the plasma used with MP was autologous MP, then the MP must be present to show a shortened $Tlag_c$ in comparison to both the MPPP and PPP samples.

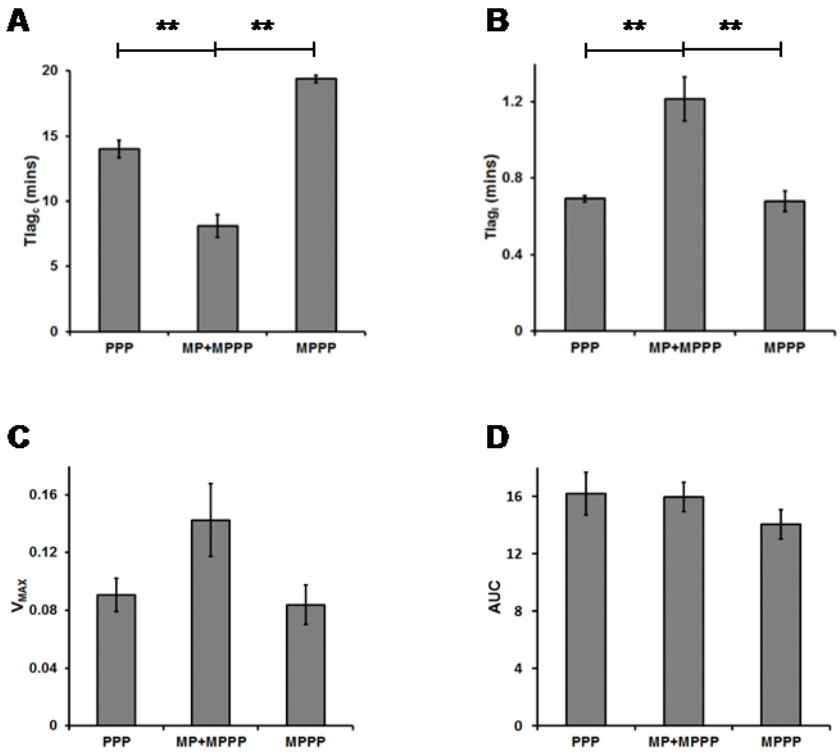
Figure 6-5 B shows a bar graph comparing the $Tlag_l$ of the MP, PPP and MPPP. This shows that $Tlag_l$ is prolonged with the MP compared to PPP and MPPP (1min 13s ± 16s *versus* 42s ± 2s *versus* 41s ± 7s). There was an increase in $Tlag_l$ with MP compared to PPP ($p < 0.01$) and MPPP ($p < 0.01$). There was no difference in $Tlag_l$ between PPP and MPPP.

The V_{MAX} and AUC were also compared between MP, PPP and MPPP (Figures 6-5 C and D). Although the V_{MAX} was higher in MP compared with PPP and MPPP (0.14 ± 0.03 *versus* 0.09 ± 0.01 *versus* 0.08 ± 0.01; Figure 6-5 C), there was no difference in V_{MAX} between them samples. There was also no difference in AUC between MP, PPP and MPPP (Figure 6-5 D).

Following this experiment, it was shown that adhered MP led to shortening of the $Tlag_c$ and prolonging of the $Tlag_l$ compared with PPP and MPPP. There was no

difference in AUC. Since there was a difference found between $Tlag_c$ and $Tlag_l$, the decision was made to use these measures to compare BS samples with HC.

Figure 6-5 Comparison of measures used in the coagulation lysis assay in samples from five healthy individuals



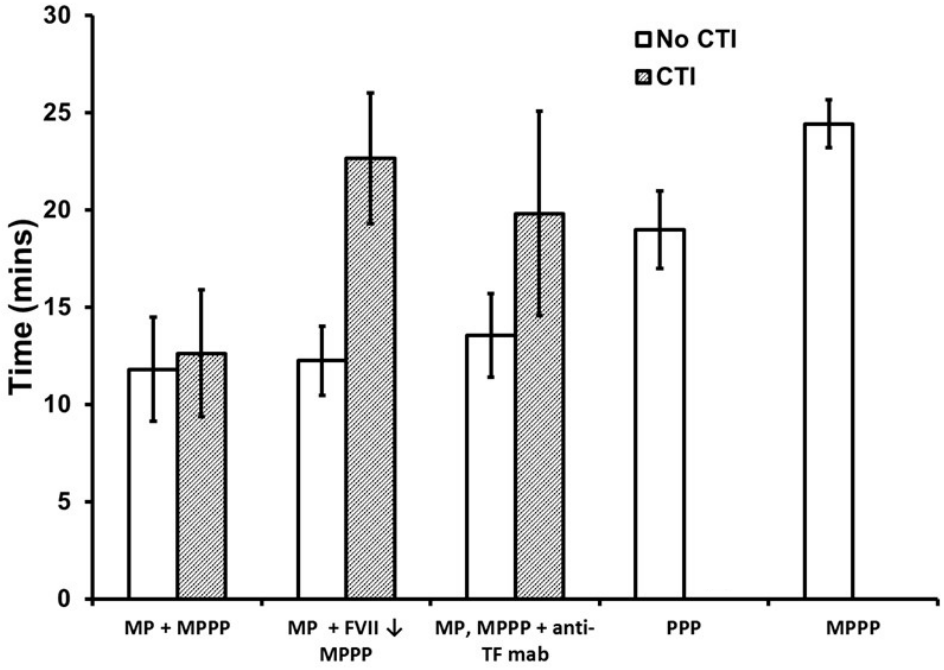
Samples were taken from five healthy individuals, with each sample run in quadruplicate. MP was prepared as previously described in section 2.6, and the plasma used in the assay with MP was autologous MPPP. The measures shown are **(A)** lag time to clot formation ($Talg_c$); **(B)** the lag time to lysis ($Tlag_l$); **(C)** maximum rate of clot formation (V_{MAX}); **(D)** area under the curve (AUC). There was a shorter $Tlag_c$ and prolonged $Tlag_l$ and increased V_{MAX} in MP compared with PPP and MPPP. **p < 0.01.

6.3.1.4 Corn trypsin inhibitor prolongs $Tlag_c$ with microparticles more than FVII deficient plasma and anti-tissue factor antibody

It was unclear why the MP led to increased coagulability, compared to autologous PPP and MPPP. A study has shown that monocyte-derived TF-expressing MP bind

to biomaterial surfaces by PS and appeared to promote increased coagulability by a TF-dependent pathway [577]. Thus, I elected to see whether this increased coagulability was due to the contact system or TF-dependent pathway (Figure 6-6). This assay had five different sample types. MP were run in three different plasmas: autologous MPPP, FVII deficient MPPP and autologous MPPP with a neutralizing monoclonal antibody to TF. Autologous PPP and MPPP were also run. Each sample was run with and without the presence of CTI. This was run in one PPP sample and were run in quadruplicate.

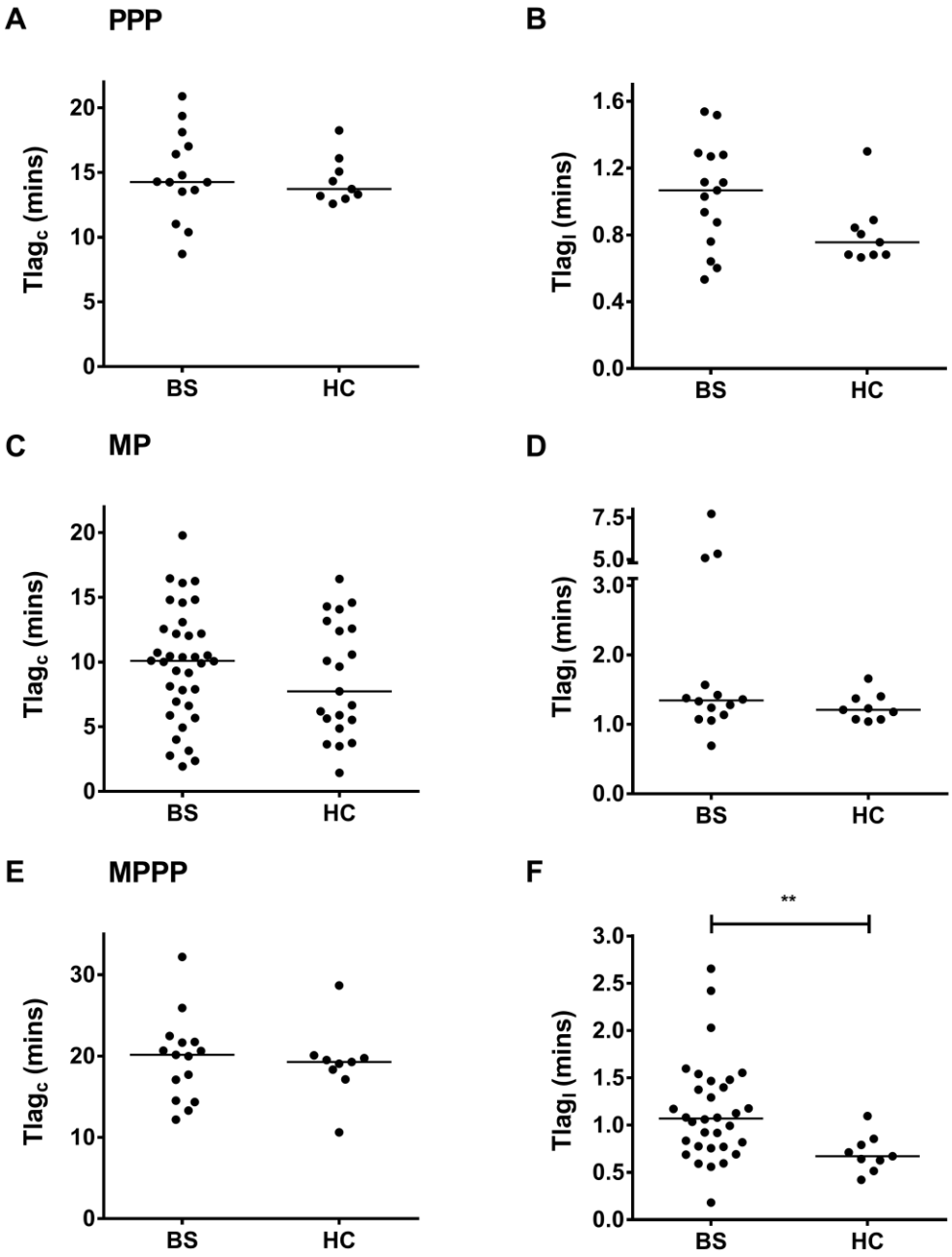
Figure 6-6 Impact of corn trypsin inhibitor, FVII-deficient plasma and neutralizing monoclonal antibody on $Tlag_c$ induced by microparticles



The sample was taken from a healthy individual and was run in quadruplicate. The columns represent the mean, with the bars representing SD. MP were run with autologous MPPP, FVII-deficient MPPP and autologous MPPP and anti-TF mAb. Autologous PPP and MPPP were run separately. Each were run with and without corn trypsin inhibitor (CTI). It was the combination of CTI with FVII-deficient plasma or anti-TF mAb that led to an increase in $Tlag_c$ with MP. CTI prevented coagulation with autologous PPP and MPPP. This suggests that it is a combination of the contact and TF-dependent pathways that lead to a decreased $Tlag_c$ in adhered MP compared to autologous PPP and MPPP.

Figure 6-6 shows that a combination of CTI and impairment of the TF-dependent pathway, either by FVII deficient plasma or neutralizing monoclonal antibody to TF, led to prolonged $Tlag_c$. Either of these on their own had no significant impact on the $Tlag_c$ of MP. CTI was confirmed as being effective by their being no clot formation by PPP or MPPP in the presence of CTI.

Figure 6-7 Comparison of lag time to clot formation and lysis in Behçet's Syndrome patients compared with healthy controls



Comparisons were made of the lag time to clot formation ($Tlag_c$) and lysis for MP, PPP and MPPP. There was no difference in $Tlag_c$ for MP, PPP and MPP between Behçet's syndrome (BS) patients and healthy controls (HC) **(A,C,E)**. $Tlag_l$ was prolonged in MPPP from BS patients compared with HC ($p < 0.01$) **(F)**, and there was no difference between BS and HC groups with PPP or MP **(B,D)**. ** $p < 0.01$.

6.3.1.5 $Tlag_c$ and $Tlag_l$ were not significantly increased in Behçet's Syndrome patients

The $Tlag_c$ and $Tlag_l$ were compared between BS patients and HC (Figure 6-7), as well as between Th+ and Th- patients (Figure 6-8). As with the studies in healthy individuals, MP, PPP and MPPP were compared and each sample was run in quadruplicate, with the mean taken. The results are presented as medians. The plasma used with MP was autologous MPPP.

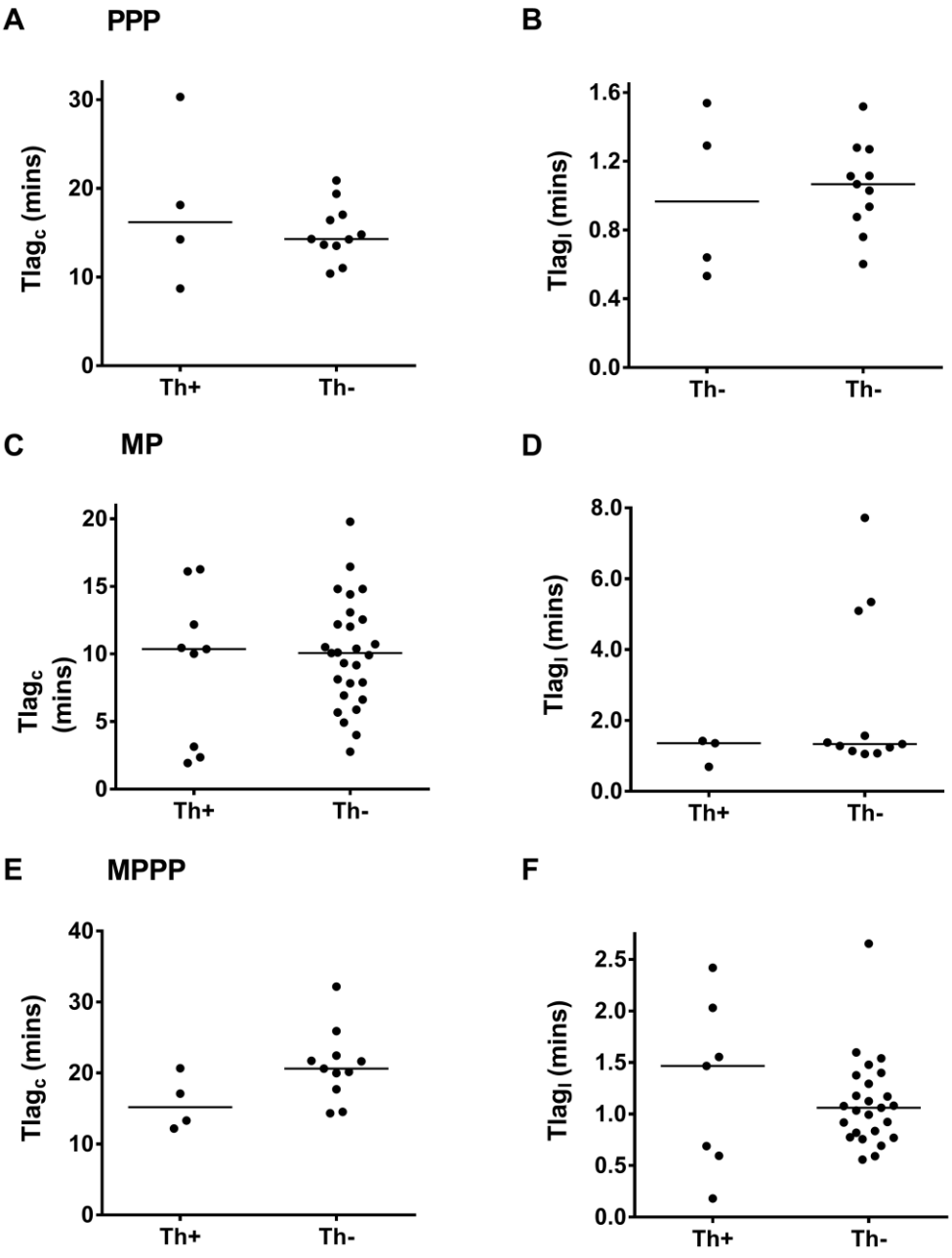
PPP from BS patients and HC were not significantly different in $Tlag_c$ (14min 16s *versus* 13min 43s; Figure 6-7 A) and $Tlag_l$ (1min 4s *versus* 45s; Figure 6-7 B). With the MP samples, there was also no difference in $Tlag_c$ (10min 5s *versus* 7min 44s; Figure 6-7 C) and $Tlag_l$ (1min 21s *versus* 1min 13s; Figure 6-7 D) between BS patients and HC. With the MPPP samples, there was difference in $Tlag_c$ (20min 9s *versus* 19min 17s; Figure 6-7 E), but $Tlag_l$ was significantly longer in BS patients compared to HC (1min 4s *versus* 46s; $p < 0.01$; Figure 6-7 F).

Figure 6-8 shows the comparison of $Tlag_c$ and $Tlag_l$ in MP, PPP and MPPP in Th+ and Th- patients. There was no difference found in either measure in MP, PPP and MPPP. With PPP, the $Tlag_c$ and $Tlag_l$ were similar (16min 11s *versus* 14min 17s and 58s *versus* 1min 4s; Figures 6-8 A and B, respectively) between Th+ and Th- patients. With MP, the $Tlag_c$ was similar between Th+ and Th- patients (10min 22s *versus* 10min 4s; Figure 6-8 C). The $Tlag_l$ with MP was also similar between Th+ than Th- patients (1min 22s *versus* 1min 2s; Figure 6-8 D). With MPPP, the $Tlag_c$ and $Tlag_l$ were also similar (15min 11s *versus* 20min 37s and 1min 28s *versus* 1min 4s; Figures 6-8 E and F).

This is not in keeping with the results shown earlier with the healthy individuals. This is despite the finding of increased TF+ MP in BS and Th+ patients compared to HC

and Th- patients, respectively, that was shown in chapter 4. At this stage, it was elected not to move forward with this assay, because there was no difference found.

Figure 6-8 Comparison of lag time to clot formation and lysis in Behçet's Syndrome patients with a history of thrombosis (Th+) and those without (Th-)



Comparisons were made of the lag time to clot formation (Tlag$_c$) and lysis for MP, PPP and MPPP. There was no difference found in Tlag$_c$ **(A,C,E)** and Tlag$_l$ **(B,D,F)** between Th+ and Th- patients.

This may be because the amount of TF+ MP produced in these disease states are not as great as in others. TF+ MP circulating in patients with malignancy are invariably tumour-derived, and the number of TF+ MP is much greater than from other conditions leading to a greater magnitude of procoagulant activity [178, 179, 307].

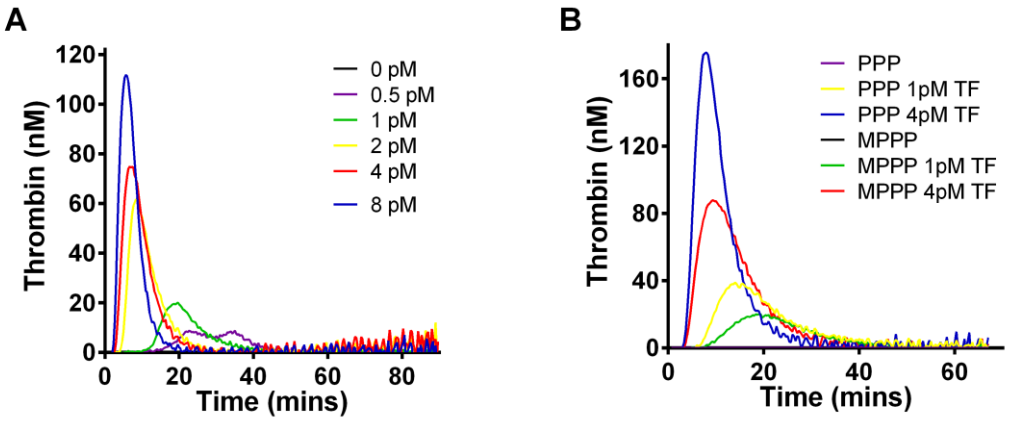
6.3.2 Calibrated automated thrombin generation assay

6.3.2.1 Tissue factor dilution series

Normal pooled PPP was collected from 10 healthy individuals. Serial PPP were run in duplicate containing 0 – 8pM TF (Figure 6-9 A). This showed that the highest peak thrombin generated was with 8pM TF, and the peak thrombin declined with decreasing concentration of TF.

In Figure 6-9 B, normal pooled PPP was compared with normal pooled MPPP from 10 healthy individuals. Serial PPP and MPPP were run in duplicate with 0 – 4pM TF. In both PPP and MPPP, there was increasing peak thrombin with increasing concentration of TF. The peak thrombin generated was highest in PPP than with MPPP. This is presumably the result of MP not being in the MPPP samples, and they provide phospholipid and TF further enhancing thrombin generation in PPP.

Figure 6-9 Thrombin generation curves from the calibrated automated thrombin generation assay and the impact of tissue factor



These curves show thrombin generation from the CAT assay. Each were the result of PPP run in duplicate. **(A)** Represents the thrombin generation curve as result of increasing TF concentration in normal pooled PPP from 10 healthy individuals. There was an increase in peak thrombin with increasing concentration of TF. **(B)** Shows a comparison in thrombin generation between normal pooled PPP from 10 healthy individuals and autologous MPPP. Both the MPPP and PPP showed increasing peak thrombin with increasing concentration of TF. The peak thrombin was highest in PPP 4pM TF with the peak thrombin in MPPP4pM TF halve that. It appears that the removal of MP has had an impact on the thrombotic potential of MPPP.

6.3.2.2 Lipopolysaccharide stimulation of healthy control blood increases peak thrombin

Peripheral blood samples were collected from healthy individuals were collected and stimulated with LPS at a concentration of 10ng/ml. Following the administration of LPS, the blood was incubated for 4 hours at 37˚C (section 2.4). As in section 2.5, PPP was prepared from the LPS-stimulated peripheral blood, aliquoted and stored at -80˚C prior to use.

In this assay, PPP, MP and MPPP samples from unstimulated and LPS stimulated healthy control peripheral blood was run in duplicate and compared (Figure 6-10). Section 2.13 documents how this assay was run, but briefly there was no exogenous phospholipid or TF added. When MP were prepared as per section 2.6, MP were resuspended to a 10µl volume with 20mM TBS, which was the equivalent

of MP from a 500µl PPP volume (section 2.13). The plasma used with the MP samples were autologous MPPP.

Overall, LPS stimulation led to increased thrombin generation in MP, PPP and MPPP compared to the unstimulated samples, which showed barely any thrombin generation (Figure 6-10). There was slightly higher peak thrombin generation in LPS stimulated PPP sample than in MP and MPPP samples (41.61nM v 28.96nM v 29.70nM, respectively), but there was thrombin generation in the unstimulated MP sample (4.17nM), but there was no generation of thrombin by the unstimulated PPP and MPPP samples.

It was expected that LPS stimulation would lead to increased thrombin generation, with the magnitude of response similar to that of TF 1pM. It was unexpected that the magnitude in response of MP from LPS-stimulated was not as high as that of PPP, because the amount of MP was the equivalent of that found in 500µl PPP. In fact, there was no difference between the thrombin generated from this MP and MPPP, which was unexpected considering the difference noted in Figure 6-8 B.

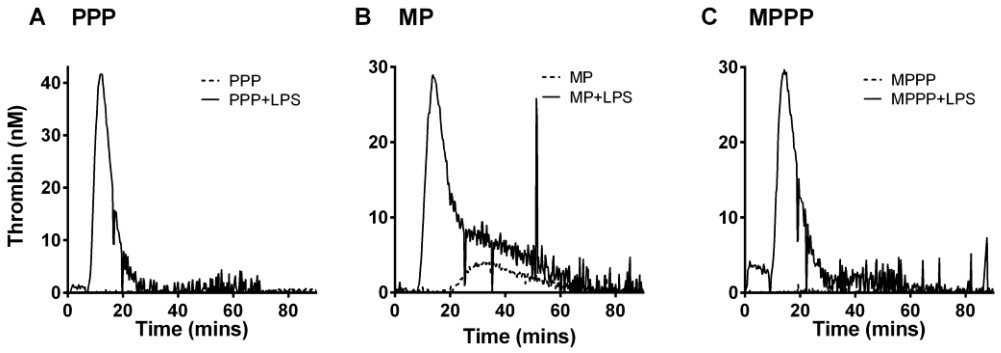
6.3.2.3 Thrombin generation in BS v HC

Thrombin generation utilising the CAT assay was compared between BS patients and HC; and between BS patients with and without a history of thrombosis (Figure 6-11). MP from the groups were prepared as before and run in duplicate. In this assay, the plasma was standardised using normal pooled MPPP prepared from 10 healthy individuals not part of the study. The MPPP was prepared as described in section 2.6. The measures from the CAT assay are as described in section 2.13.

Median ETP was increased in BS patients compared with HC (554.7nM v 360.2nM; $p < 0.05$; Figure 6-11 A), as was median peak thrombin (20.28 v 10.13; $p < 0.01$;

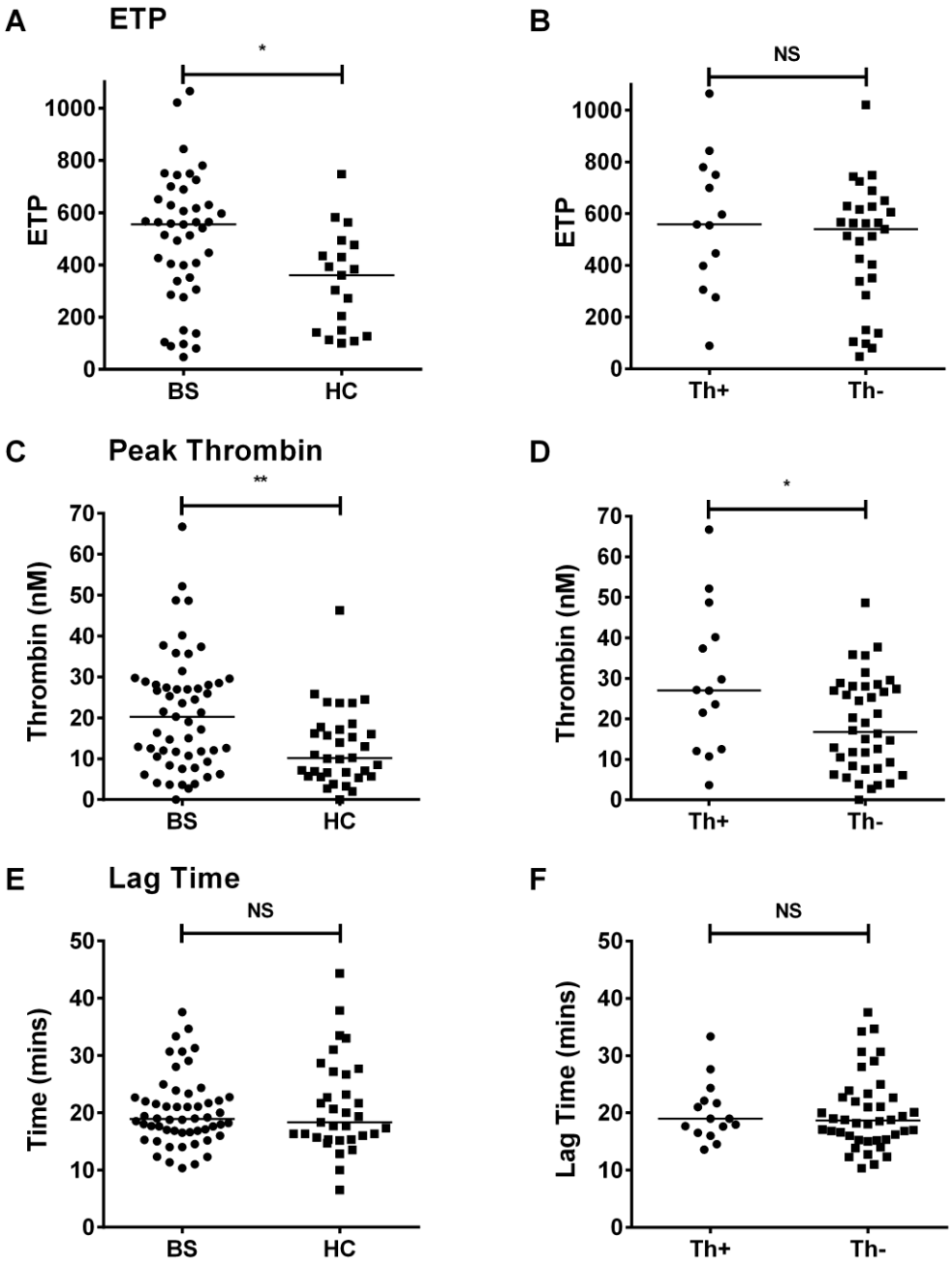
Figure 6-11 C). Lag time to thrombin generation was not significantly different between BS patients and HC (Figure 6-9 E). Median peak thrombin was increased in the Th+ group compared with the Th- group (27.06nM v 16.73nM; $p < 0.05$; Figure 6-11 D). There was no difference in median ETP or lag time to thrombin generation between Th+ and Th- patients, and was almost identical between the two groups (Figures 6-9 B and F).

Figure 6-10 Thrombin generation curves comparing PPP, MP and MPPP from LPS-stimulated and unstimulated whole blood



Whole blood was incubated with LPS at a concentration of 10ng/ml and incubated at 37˚C for 4 hours to activate cells and generate MP. Following this step, PPP was prepared as previously described in section 2.5 and aliquoted and stored at -80˚C. PPP, MP and MPPP were run, with samples from stimulated whole blood will be suffixed '+LPS' and compared with samples from unstimulated whole blood. All samples were run without the addition of exogenous TF or phospholipid. **(A)** shows the thrombin generation curves for PPP+LPS and unstimulated PPP. There was thrombin generated from PPP+LPS, but none from the PPP. **(B)** MP+LPS was higher than unstimulated MP, but there was thrombin generated from the unstimulated MP. **(C)** shows thrombin generation MPPP+LPS compared to unstimulated MPPP. There was thrombin generated from MPPP+LPS, but none from unstimulated MPPP. Samples were run in duplicate.

Figure 6-11 Assessment of thrombin generation of microparticles from Behçet's Syndrome patients and healthy controls, as well as BS patients with a history of thrombosis (Th+) and those without (Th-)



The CAT assay was used to assess MP thrombin generation. The assay was used to compare MP from BS patients and HC, and Th+ and Th- patients. The measures were endogenous thrombin potential (ETP) **(A, B)**, peak thrombin **(C, D)** and lag time to thrombin generation **(E, F)**. There was an increased ETP in BS patients compared with HC **(A)** and increased peak thrombin in BS and Th+ patients, compared with HC and Th- patients, respectively. *p< 0.05 **p< 0.01, NS = non-significant

6.3.2.4 Correlation between thrombin generation and MP counts

Multiple correlations were done between ETP, peak thrombin and lag time to thrombin generation and total, CD14+, TF+ and CD14+ TF+ MP. There was no significant positive correlation found between any of the thrombin generation measures and MP counts in HC or BS, Th+ and Th- patients (Appendix 2).

6.3.3 Zymuphen MP-TF assay

The MP-associated TF activity was measured by the Zymuphen MP-TF kit as documented in section 2.14. This kit has been shown to measure the amount of TF in plasma and assess the procoagulant activity of plasma cell derived TF-bearing MP in the literature [309, 310]. Each patient sample was run in triplicate and the assay was run twice. Standard curves were done each time the assay was run and showed a linear response with increasing TF concentration, there was increasing FXa generation (Figure 6-12).

Figure 6-12 Standard curves for the Zymuphen MP-TF assay

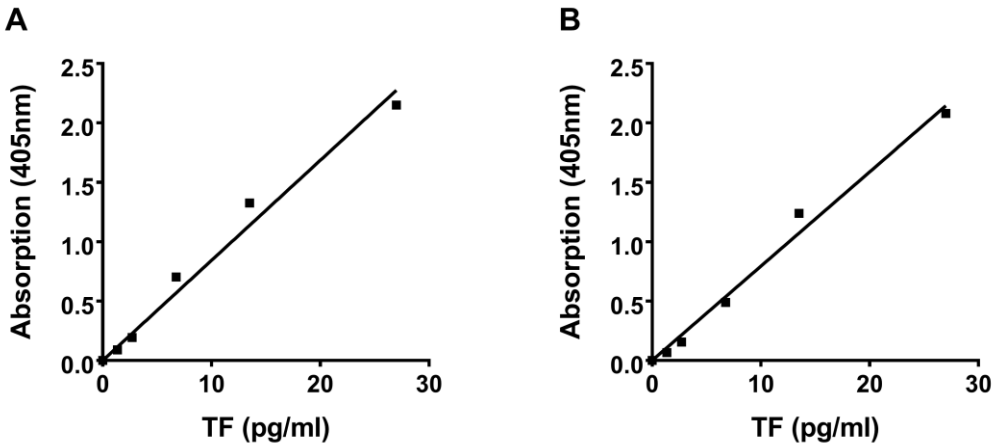
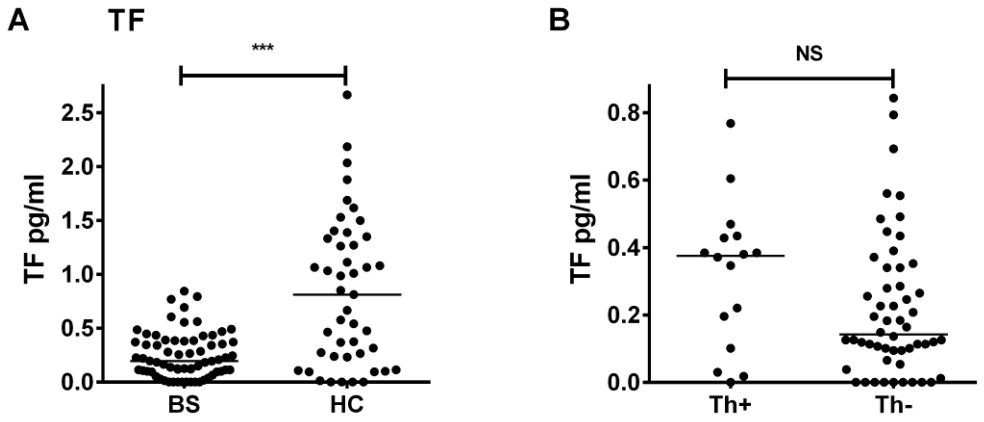


Figure **(A)** and **(B)** shows the standard curves the FXa generation assay. This assay was run twice and each sample was run in duplicate. The standard curve was calculated as per the manufacturer's guidelines. There was consistency between the two curves suggesting reproducibility.

Figure 6-13 shows the TF measured from the BS patients compared with HC and in the Th+ compared with the Th- patients. MP were prepared as per section 2.6 and protocol followed the manufacturer's instructions. The median TF measured in BS patients was decreased compared to that in HC (0.20pg/ml *versus* 0.81pg/ml; $p < 0.0001$; Figure 6-12 A), whilst there was no difference in TF measured between Th+ and Th- patients (0.38g/*ml versus* 0.14pg/ml; Figure 6-13 B).

In this assay, the normal range for TF concentration was less than 2pg/ml, so none of the results were greater than that. It was surprising that the TF concentration of BS patients was so low, and, based upon the standard curves, the assay was run correctly. This would suggest that there are other factors within the plasma that was inhibiting the generation of FXa.

Figure 6-13 Comparison of tissue factor levels in Behçet's Syndrome patients compared with healthy controls, and in Behçet's Syndrome patients with a history of thrombosis (Th+) and those without (Th-) using the Zymuphen MP-TF assay



The assay was run as per manufacturer's guidelines, so 20µl plasma was added to each well. **(A)** shows that the median TF was greater in the HC group than the BS patients, but both were < 2pg/ml, which was within normal range. **(B)** showed no difference between Th+ and Th- patients.

6.4 Discussion

6.4.1 The coagulation assays did not consistently show difference between BS patients and HC, or Th+ and Th- patients

The use of MP in coagulation lysis, CAT and factor Xa generation assays did not consistently demonstrate significant differences between BS patients and HC or between Th+ and Th- patients. In the study by Eleftheriou, the CAT assay demonstrated increased thrombin generation by MP from patients with active childhood vasculitis and a history of thrombosis, compared to those without a history of thrombosis [343]. Only one patient had BS, who had active vasculitis and cerebral sinovenous thrombosis. Otherwise, there have been no studies that have shown a coagulation test that identifies increased thrombotic risk in those with BS.

The coagulation lysis assay showed decreased $Tlag_c$, prolonged $Tlag_l$ and increased V_{MAX} in MP compared to PPP and MPPP in 5 healthy individuals (Figure 6-5). This showed that MP annealed to the well surface shortened the $Tlag_c$ compared to remaining in suspension in plasma. The $Tlag_c$ was shortened compared to autologous PPP in BS and HC samples (Figure 6-7). It has been shown that monocyte-derived MP that express TF, adhere to biomaterial surfaces and promote coagulation not just by the intrinsic pathway, but by a TF-dependent pathway [577]. In Figure 6-6, it was blockade of both the contact system by CTI and the TF-dependent pathway, either by FVII-deficient plasma or a neutralising monoclonal antibody to TF, that led to an increase in $Tlag_c$ by MP. It remains unclear why this should be particularly the case, since coagulation by PPP was completely abrogated by CTI. This suggests that there is further surface rearrangement of MP following their adherence of MP to the biomaterial surface.

The prolonged $Tlag_l$ suggests that the clot formed by MP was more resistant to thrombolysis. Increased TF+ MP have been shown to be associated with resistance to thrombolysis in the treatment of acute myocardial infarction and failure of recanalization [640]. This was only found in Figure 6-5, but was not found in BS and HC samples.

6.4.2 Increased reteplase concentration impairs lysis

Figures 6-2 and 6-3 demonstrate a reteplase titration series looking for the appropriate dose of reteplase to induce fibrinolysis in the coagulation lysis assay. As the reteplase concentration approaches 0.1mU/100µl plasma, fibrinolysis was induced, whilst increasing the dose beyond 1mU/100µl plasma impaired fibrinolysis. This was just below the calculated concentration of reteplase when it is used in the clinical situation.

Reteplase and other recombinant tissue plasminogen activators paradoxically activate the intrinsic system, with increased thrombin generation [641, 642]. Thus, following reteplase administration, antiplatelet agents such as abciximab, a platelet glycoprotein IIb/IIIa receptor antagonist, and heparin are used to reduce the risk of thrombosis [643, 644]. It is also though that higher concentrations of reteplase lead to proteolytic cleaving of plasminogen preventing lysis of the fibrin clot [578]. Reteplase at high concentrations leads to flooding of fibrin receptors, preventing the binding of plasmin to fibrin, which is necessary for fibrinolysis of the clot [645]. Consequently, heparin infusion commonly follows retaplase therapy in clinical practice.

6.4.3 Lag times to clot formation does not discriminate between microparticles from Behçet's Syndrome patients and healthy controls

In neither the coagulation lysis nor the CAT assay, was $Tlag_c$, sensitive enough to identify a difference between any of the groups. The $Tlag_c$ represents two distinct aspects of the coagulation cascade in these assays. The $Tlag_c$ in the coagulation lysis assay measures the increase in turbidity, which is due to the formation of fibrin, whilst it measures time to thrombin generation in the CAT assay. It is possible that as the $Tlag_c$ becomes shorter, the differences diminish, such that large cohorts are required to detect a difference. This suggests that this measure is not sensitive enough to detect differences in TF+ MP levels as found in the BS cohort.

6.4.4 Peak thrombin discriminates between Behçet's Syndrome patients and healthy controls and is increased in Behçet's Syndrome patients with a history of thrombosis compared to those without

The peak thrombin measured was significantly higher in BS and Th+ groups compared to HC and Th- groups respectively. The median thrombin generated in Figures 6-11 C and D in BS and Th+ groups, respectively, was equivalent to that from PPP with addition of 1pM TF. This is even in the context of no additional corn trypsin inhibitor (CTI). CTI is a FXIIa inhibitor, and inhibits the contact activation of the coagulation cascade [646]. Thus, there was no impediment to optimum thrombin generation and both the TF and contact activation pathways were active in thrombin generation. The CAT assay was not effective in identifying the difference between BS and HC groups, or between Th+ and Th- groups.

The changes found in this assay could have been investigated further. It was not clear why there was so little TF generation, considering the higher TF+ MP counts found in BS and Th+ patients compared to HC and Th- patients found in chapter 4. It was shown in Figure 6-9 B, that there was more thrombin generated from PPP than MPPP with equivalent TF concentrations. This appeared to be the result of removal of MP, which could have led to a reduction in TF or phospholipid surface for coagulation to occur on. The other possibility is that there was increased level of TFPI, which could have been assessed by an assay.

6.4.5 No correlation between CAT measures and MP count

There was no correlation found between MP count, particularly TF+ MP, and CAT measures. This may be because there are other factors that inhibit coagulation in this group of samples. As mentioned, studies have shown an increased level of TFPI in BS patients, which are thought to be a reactive measure and relate to increased risk of thrombosis [554, 647]. It is possible that TFPI may be present to block the effect of TF in inducing thrombin generation.

6.4.6 Microparticle associated tissue factor generation was not raised in Behçet's Syndrome patients

The Zymuphen MP-TF assay was used to identify the concentration of TF+ MP in samples. Studies have demonstrated its efficacy and sensitivity in conditions associated with raised TF+ MP levels, although it may not be as sensitive as the CAT assay [308]. It was thought that this could be a useful assay to assess the TF dependent pathway. In Figure 6-13 A, the TF concentration was found to be higher in HC than BS patients. This was an unexpected finding, however, the TF

concentrations found were below the upper limit of normal for the assay. It is possible that there are other aspects within the plasma of BS patients that impair TF activity. This could be TFPI, which is known to complex with FXa, and then form a quaternary complex with TF and FVIIa preventing activation of the TF-independent pathway. To show this, it would have been useful to add in a monoclonal antibody to TFPI to see if this would lead to an increase in TF activity. Blocking TFPI has been shown to increase the sensitivity of the thrombin generation assay, and may explain why the TF+ MP count is a poor

It is also possible that there are not enough TF+ MP in BS patient PPP to generate enough FXa. FXa generation has been shown to be increased in patients with malignancy associated with thrombosis, such as pancreatic and metastatic breast cancers, and in murine models of thrombosis [178, 304, 306]. There is often a much greater number of TF+ MP in patients with malignancy, because they are directly from the tumour [317, 320]. This suggests that this assay is more sensitive in assessing TF concentration in those conditions than in others.

This lack of correlation between the results of the coagulation studies and AnV-binding and TF+ MP, maybe the result of the recognised TF being latent on the surface of MP, and is only activated in areas of inflammation when encountering fibrin and neutrophil extracellular traps [597]. Preclinical models show that protein disulphide isomerase de-encrypts surface TF on MP thereby promoting coagulation and thrombosis [326, 648, 649]. This would explain why the basal coagulation activity is low in these groups, yet there is raised circulating MP and MP expressing TF.

7 Tissue Factor Pathway Inhibitor and Microparticles in Behçet's Syndrome

7.1 Background

TF is the most powerful activator of the coagulation cascade and increases the catalytic ability of FVIIa to convert FX to FXa by 2-million-fold [42]. TFPI is a large Kunitz-type glycoprotein that inhibits the function of TF-FVIIa complex and FXa, thereby being the primary inhibitor of the blood coagulation cascade [72]. There are two major isoforms of TFPI: α and β. The α and β isoforms differ in structure, with the α isoform having three tandem Kunitz-type domains with a C-terminal, whilst the β isoform has two Kunitz-type domains, a differing C-terminal and a glycosylphosphatidylinositol anchor to the membrane (Figure 1-2) [72]. The Kunitz-2 domain interacts with FXa, whilst the Kunitz-1 domain inhibits the TF/FVIIa complex in a FXa-dependent manner [76, 77]. The α isoform circulates free or is bound to lipoproteins [84]. The β isoform is the most abundant and attaches to cell membranes via a glycosylphosphatidylinositol anchor [85]. Endothelial cells and megakaryocytes primarily produce TFPI, although it is also synthesised by other cell types such as monocytes [72, 73, 86].

It has been shown that blocking TFPI enhances the thrombin generation potential of MP [90]. There is a suggestion that reduced TFPI activity may lead to recurrent venous thromboembolism in the general population, increased procoagulant state in pregnancy and increased monocytic procoagulant activity in acute myocardial infarction [650-653].

Conversely, TFPI levels have been shown to be increased in BS, but the difference between thrombotic and non-thrombotic patients was not addressed [647]. There is also evidence that heparin does not mobilise TFPI in BS as in other conditions,

suggesting that there are reduced endothelial stores of TFPI or that they are resistant to mobilisation [554]. It has also been demonstrated that TFPI regulates TF activity on the surface of monocytes, thereby ameliorating their procoagulant effect [654].

Results from Chapters 4, 5 and 6 showed that although there were increased levels of total and TF+ MP in BS, this did not translate into functional significance using in vitro coagulation assays except with the TF assay. The CAT assay showed increased peak thrombin generation in BS, but the FXa generation assay showed the TF dependent pathway was not responsible. This may be because the critical mass of TF+ MPs required for significant functional impact does not occur in BS, as it does in malignancy, or there may be an alternate process blocking the pro-thrombotic effect of TF+ MP.

Herein, I investigated whether there was a difference in TFPI+ MP counts between BS patients and HC and how any differences might correlate to varying quantities of TF+ MP.

7.2 Methods and Materials

7.2.1 Peripheral blood samples

Samples were collected from healthy controls and BS patients recruited into the study, from which PPP was prepared as per sections 2.2 – 2.5. Patient and healthy control details are as documented in Table 4.1. PPP were stored as 500µl aliquots at -80˚C awaiting analysis (see section 2.5 for further details).

7.2.2 Microparticle preparation

MP were prepared from PPP samples as per section 2.6. Once prepared samples were resuspended to 500µl in 1 X annexin V buffer and stored on ice ready for mAb staining.

7.2.3 Conjugation of fluorochrome to anti-TFPI K1 antibody

MAb was conjugated to AF-647 Apex® molecular beads as per section 2.10. Briefly, 10µl (20µg) of anti-TFPI K1 mAb was conjugated to the beads, and, once complete, the conjugated mAb was stored at 4˚C ready for use. The isotype control mAb underwent the same procedure. This avoided the use of a secondary antibody, which would necessitate a further 17000g x 60min centrifugation step. The problem with further centrifugation steps is aggregation of MP [218].

7.2.4 Preparation of HUVECs and preparation of HUVEC-derived microparticles

Human umbilical vein endothelial cells (HUVECs) express TFPI and were used to test the efficacy of the conjugated anti-TFPI K1 mAb, as detailed in section 2.11.

7.2.5 Flow cytometry

Flow cytometry was performed on both HUVEC and HUVEC-derived MP, as previously described in section 2.11. HUVEC were stained with AF-647-conjugated anti-TFPI K1 mAb and were incubated for 30 minutes at 4˚C in the dark. MP were dual stained with PB Annexin V and AF-647-conjugated anti-TFPI K1 mAb and incubated at room temperature in the dark. AF-647-conjugated IgG1 isotype control was at the same concentration used in all experiments. The mAb was also

incubated with 800ng recombinant human TFPI (gratefully received from J. Ahnstrom and purified as per Ahnstrom *et al*, Blood 2012), which was there to competitively inhibit the mAb to show there was no non-specific binding. The TFPI was added to the HUVECs suspension and the AF-647 conjugated anti-TFPI K1 mAb was added as before.

7.2.6 Statistical analysis

All results are quoted as medians with statistical comparisons accomplished by Mann-Whitney U test (see section 2.15 for further details)

7.3 Results

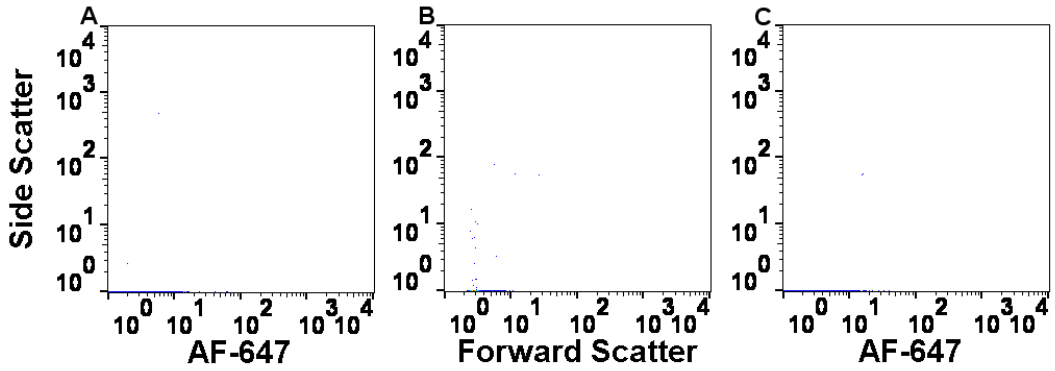
7.3.1 Efficacy of conjugated antibody with HUVECs

Prior to any assessment of the conjugated mAb with HUVEC or HUVEC-derived MP, the mAb bound to the molecular beads were run in the flow cytometer to make sure there was no signal that would impact on analysis. Figure 7-1 shows a comparison between the negative control, molecular grade water, and mAb resuspended in molecular grade water. Figure 7-1 A showed there were no events seen in the side *versus* AF-647 plot. Figure 7-1 B there was no events found for AF-647 beads in the side *versus* forward scatter plot, nor were they seen in the side versus AF-647 plot. This showed that the beads were not detectable by this flow cytometry protocol.

Conjugated AF-647 anti-TFPI K1 mAb was assessed for its efficacy with HUVEC and HUVEC-derived MP (Figure 7-2). In all samples, HUVEC was present. The comparison was between AF-647-conjugated anti-TFPI mAb and 800ng recombinant human TFPI, AF-647 IgG1 isotype and unstained HUVEC (Figure 7-2 A). The MFI was 9.73 with mAb and HUVEC, whilst blocking of the antibody with

800ng TFPI, IgG1 isotype control and no mAb had similar MFI (3.29 v 3.18 v 3.06). This shows that the mAb bound to TFPI expressed by HUVEC and was blocked by recombinant human TFPI. There was no signal from the isotype control or from the HUVEC themselves.

Figure 7-1 Flow cytometry of the anti-TFPI K1 monoclonal antibody conjugated to the AF-647-conjugated APEX® molecular beads

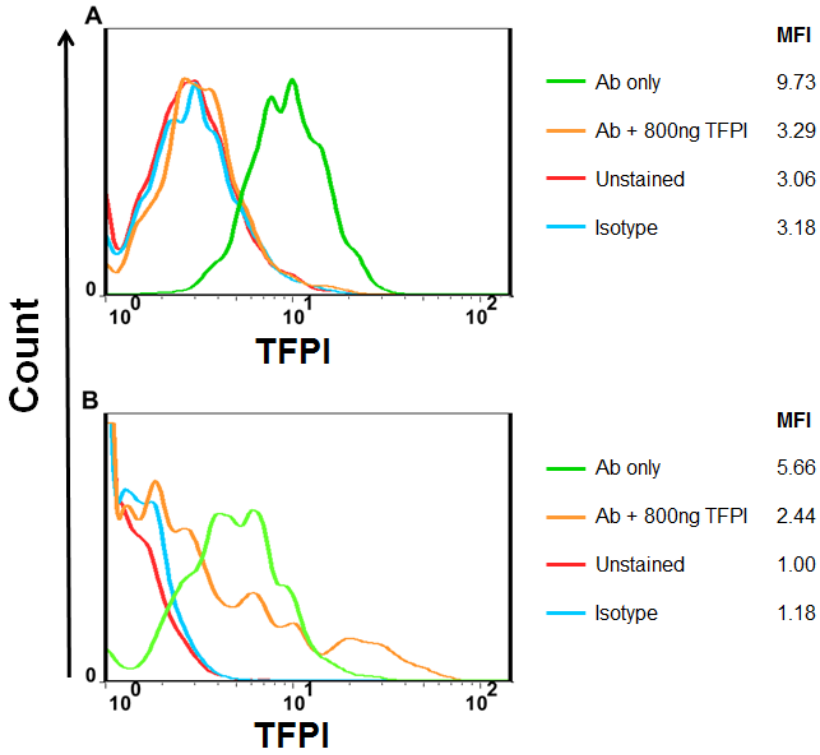


This figure shows dot plots following the run of the AF-647 molecular beads to see if there were any notable events seen. **(A)** Molecular grade water only was run as a negative control and did not show numerous events in the side scatter *versus* AF-647 plot. **(B)** The AF-647-conjugated anti-TFPI K1 mAb was suspended in molecular grade water and run through the flow cytometer. The events seen in this plot was typical of that seen with molecular grade water (see Figure 3-3 C and D). **(C)** There was no increase in events in the side scatter *versus* AF-647 plot.

7.3.2 Efficacy of conjugated antibody with HUVEC-derived microparticles

Conjugated AF-647 anti-TFPI K1 mAb was also incubated with HUVEC-derived MP (Figure 7-2 B). As with HUVECs, the MFI was measured and comparison was made between AF-647 conjugated anti-TFPI K1 mAb and 800ng recombinant human TFPI, IgG1 isotype control and unstained HUVEC-derived MP. The MFI was 5.66 with mAb, whilst blocking of the mAb with 800ng TFPI was 2.44, AF-647 isotype 1.38 and unstained was 1.00.

Figure 7-2 Assessment of AF-647-conjugated anti-TFPI K1 monoclonal antibody with HUVEC and HUVEC-derived microparticles



Assessment of AF-647-conjugated anti-TFPI antibody (Ab) was performed with HUVEC **(A)** and with HUVEC-derived MP **(B)** using flow cytometry. In both cases, HUVEC or MP were incubated with Ab, Ab and 800ng TFPI, IgG1 isotype control or left unstained. All results are given as MFI. **(A)** The MFI shifted to the right with HUVEC bound to Ab, whilst the Ab and 800ng TFPI, isotype and unstained samples remained the same. **(B)** The MFI did not shift to the right with the MP as with HUVEC. The MFI of the Ab and 800ng TFPI, isotype and unstained are as documented. There was still staining by Ab in the Ab and 800ng TFPI sample of MP, showing it was not completely abrogated by TFPI. MFI = median fluorescence intensity.

Overall the results with HUVEC and HUVEC-derived MP showed that the mAb did specifically bind TFPI, and was blocked by recombinant TFPI. This was not as clear with the MP, but it did seem to show that there was partial, but not complete blockade of the binding of mAb to MP by TFPI. It is known that larger cells will bind more mAb than smaller ones, making them more fluorescent, and MP have a smaller surface area for binding of mAb. This is represented by median fluorescence and is shown by the larger MFI of HUVEC bound to mAb than HUVEC-derived MP, as well as the MFI of unstained HUVEC (Figure 7-2). In MP

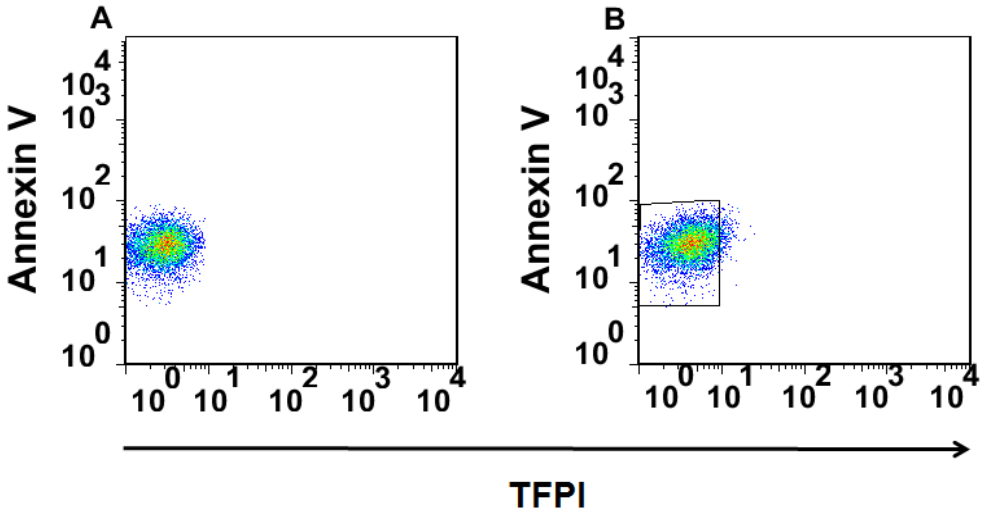
flow cytometry, the gate is set such that all particles less than 1.1μm in diameter are included in the analysis. This means that the histograms show a longer tail than those of cells. Based upon Figure 7-2 B, the 800ng recombinant human TFPI did not completely block the mAb, but it did partially block it, when compared to the HUVEC-derived MP only. Based on this, we felt it reasonable to proceed with further experiments utilising the mAb.

7.3.3 Assessment of TFPI-bearing microparticles

Figure 7-3 shows the gating strategy to detect TFPI+ MP, comparing the IgG1 isotype control with the TFPI-specific mAb. This assay was performed using HUVEC-derived MP. As is shown, there is a shift to the left of the MP in fluorescence when the MP bind mAb, which is not seen with the isotype control. The gate was drawn around the isotype and all those events that fall to the right of this were MP less than 1.1μm in diameter, binding annexin V and positively expressing TFPI.

Figure 7-4 shows a comparison of TFPI+ MP in count and proportion between BS patients and HC (Figures 7-4 A and B, respectively) and in BS patients with and without a history of thrombosis (Figures 7-4 C and D, respectively). The median count (3.6×10^4/*ml versus* 2.0×10^3/ml; $p < 0.001$; Figure 7-4 A) and proportion (5.2% *versus* 0.9%; $p < 0.001$; Figure 7-4 B) of TFPI+ MPs are increased in BS patients compared to HC. There was no difference in TFPI+ MP median count (3.1×10^4/ml *versus* 3.7×10^4/ml; Figure 7-4 C) between Th+ and Th- patients, but there was a decreased median proportion TFPI+ MPs in Th+ than in Th- patients (2.6% *versus* 5.9%; $p < 0.05$; Figure 7-4 D).

Figure 7-3 Gating strategy for AF-647-conjugated TFPI K1 monoclonal antibody, using HUVEC-derived microparticles



IgG1 isotype control **(A)** compared with AF-647-conjugated anti-TFPI K1 mAb **(B)**. Gate in **(B)** denotes the IgG1 isotype control, with MP outside this gate defined as TFPI+ MP and these bind AnV.

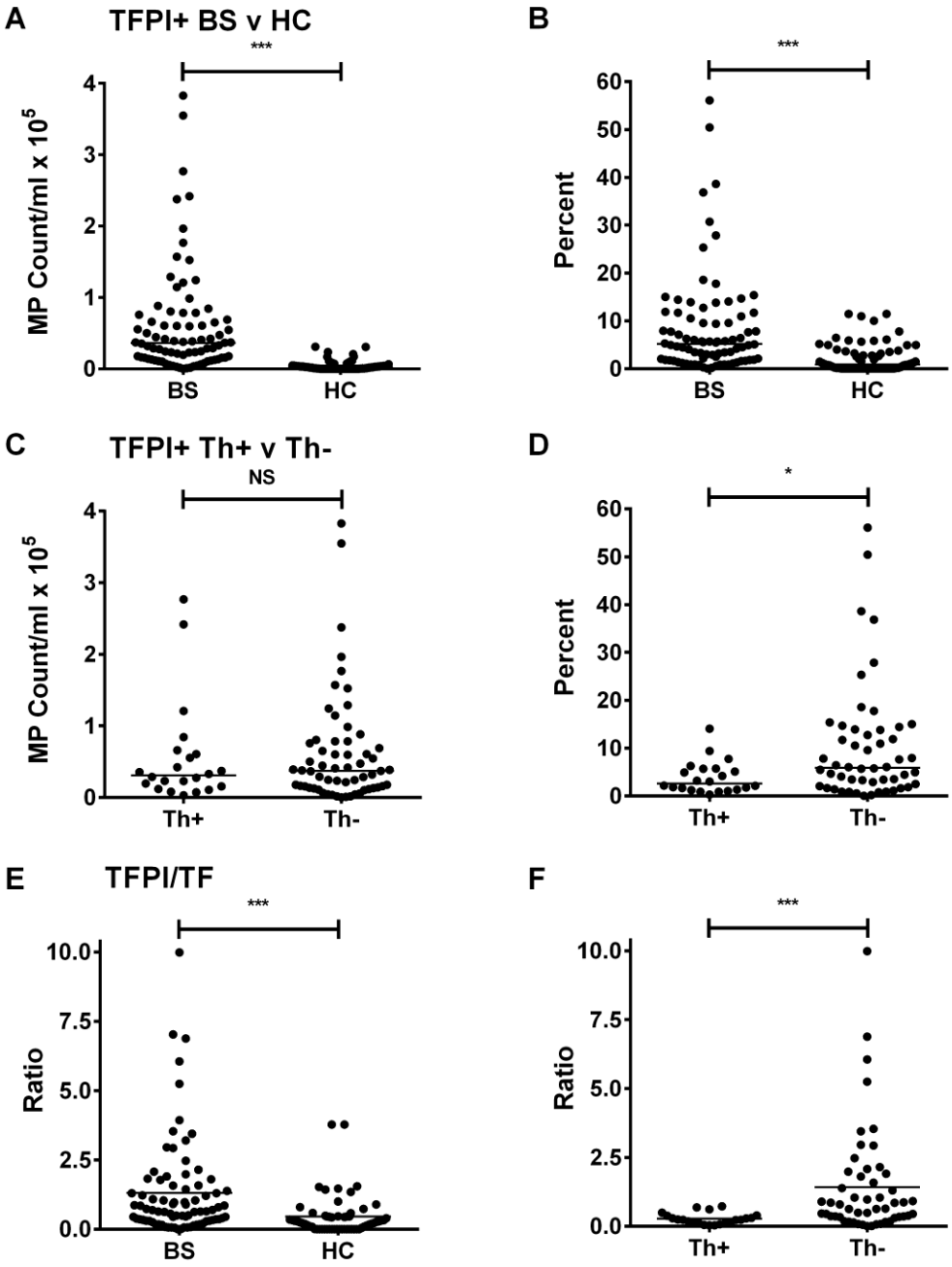
7.3.4 TFPI+ MP/TF+ MP (TFPI/TF) ratio

The balance of TF and TFPI activity in the circulation is important to maintain the integrity of haemostasis. The balance of TF+ and TFPI+ MP has been investigated in pregnant women, and an increased TF/TFPI ratio was found in women with gestational vascular complications, compared to those without gestational vascular complications [652]. Figure 6-13 A also showed the median TF activity was reduced in BS patients compared with HC. Thus, I investigated whether there was an increase in TFPI+ MP/TF+ MP count (TFPI/TF) ratio to explain this.

Figure 7-4 E and F shows the TFPI/TF ratio in BS patients compared with HC and in BS patients with a history of thrombosis compared to those without. The median TFPI/TF ratio was calculated in all groups. The TFPI/TF ratio in BS patients was greater than in HC (0.67 *versus* 0.25; $p < 0.001$; Figure 7-3E). The greatest contribution to the TFPI/TF ratio in BS patients came from BS patients without a

history of thrombosis. The TFPI/TF ratio was increased in BS patients without a history of thrombosis compared to those with (0.72 *versus* 0.23; $p < 0.001$; Figure 7-3F). There were no BS patients with a history of thrombosis who had a TFPI/TF ratio greater than or equal to 0.7 (χ^2, $p < 0.0001$).

Figure 7-4 Comparison of TFPI-bearing microparticles in Behçet's Syndrome with healthy controls, and in Behçet's Syndrome patients with (Th+) and without (Th-) thrombosis, and their relationship to TF-bearing microparticles



Circulating TFPI+ MP are increased in BS but relatively low in patients with a history of thrombosis. MP were enumerated by flow cytometry using Annexin V and anti-TFPI mAb. **(A, B)** TFPI+ MP in BS *versus* HC expressed as absolute counts **(A)** or % total MP counts **(B)** ; TFPI+MP in Th+ *versus* Th- expressed as absolute counts **(C)** or % total MP counts **(D)**; (**E,F**) ratios of TFPI to TF counts in BS *versus* HC **(E)** and Th+ *versus* Th- patients **(F)** *** $p < 0.001$, * $p < 0.05$, NS = non-significant.

7.4 Discussion

7.4.1 Detection of TFPI on microparticles with AF-647 Apex® molecular beads conjugated with anti-TFPI K1 monoclonal antibody

I was able to show that binding of anti-TFPI K1 mAb to AF-647 Apex® molecular beads was an effective method to perform MP flow cytometry to detect surface TFPI. In other studies, TFPI+ MP were detected either with a polyclonal antibody or a mAb and secondary antibody [652, 655]. The former suffers from lack of specificity, whilst the latter involves further centrifugation steps, which risks aggregation of MP affecting counts [218].

By using the Apex® molecular beads to conjugate the anti-TFPI K1 mAb, those pitfalls were avoided. There was no necessity for a washing step following binding. It also meant that the experimental conditions remained the same as those in chapters 4 and 5. An issue with using these beads was concern that the beads would be seen in plots, but this was not the case (Figure 7-1). There is little literature showing its use, but it has been used for mAb labelling for fluorescence spectroscopy in epigenetics research [656]. The main flaw is that it is presumed when labelling 20µg of mAb that all mAb is labelled, but it is unclear whether that is the case. This is a solid-phase labelling kit, to which the mAb binds covalently. Atomic force microscopy has shown these to be strong bonds, so it is unlikely that there would be detachment of the mAb [657], but it is unclear how much mAb binds initially.

7.4.2 TFPI-bearing microparticles are increased in Behçet's Syndrome patients compared with healthy controls

Herein, TFPI+ MP have been shown to be increased in BS patients compared with healthy controls. There was an increased proportion of TFPI+ MP in Th- patients than Th+. In chapter 6, there was a discrepancy shown between the functional and flow cytometric data. TFPI on the surface of cells has been shown to impair TF-FVIIa activity on the surface of breast cancer cells, confirming its potency in blocking the extrinsic coagulation cascade, and TFPI+ MP have been shown to impair the thrombogenicity of TF+ MP in pancreatic cancer in an in vivo model [658, 659].

The observation that the overall TFPI+ MP in BS is raised, is consistent with the result of ELISA studies in BS patients showing raised TFPI activity [660]. There was no TFPI ELISA done in this study, so there was no comparison between raised TFPI+ MP and increased TFPI function. It is possible that the rise in TFPI activity in BS patients is a homeostatic adaptation to reduce the risk of thrombosis in the inflammatory setting [660]. Within BS patients there was no demonstrable difference between Th+ and Th- groups, although the proportion of TFPI+ MP was lower in Th+ than Th- patients. It is possible that a greater inflammatory environment in the Th+ group results in more TFPI degradation by leukocyte-derived serine proteases and/or reactive oxygen species [661, 662]. This would lead to a decrease in TFPI relative to TF

7.4.3 TFPI/TF ratio may identify patients at risk of thrombosis

Although these results do not identify which cell-derived MP express TFPI, the implication of the result shown in Figure 7-4 F is that the balance between TFPI+

MP and TF+ MP may be associated with an increased risk of thrombosis in this cohort of BS patients. This association is shown by Th- patients having a higher TFPI/TF ratio than Th+ patients, and no patients with a TFPI/TF ratio greater than 0.7 had a history of thrombosis. This result would need to be validated in other cohorts of BS patients, before it could be seen as a definitive cut off to identify increased risk of thrombosis. The reasons as to why we found some BS patients have a relatively low level of MP-associated TFPI compared to MP-associated TF are unclear, but could include differential synthesis of TF and TFPI by activated monocytes [87], TFPI degradation by leukocyte-derived serine proteases [662, 663] and/or reactive oxygen species and the influence of genetic variants of TFPI on plasma levels [664].

There is a value to the TFPI/TF ratio, but it is diminished by a lack of support from the results of functional studies. As mentioned in chapter 6, the use of anti-TFPI mAbs can lead to increased sensitivity of coagulation assays, which may have revealed an increased thrombotic tendency [90]. It has been shown that the inverse of this relationship, TF/TFPI, has been shown to be increased in pregnant women and those gestational vascular complications [652]. Cord blood plasma is associated with an increased risk of thrombosis, associated with an imbalance in TF to TFPI activity, whilst maternal blood plasma demonstrates the inverse of this relationship [665].

8 Overall Discussion

8.1 Summary of Results

In the work for this thesis, my aim was to establish whether MP were increased in BS patients, particularly those with a history of thrombosis. First, flow cytometry was performed to enumerate and characterise MP. These were characterised as <1.1μm in diameter using beads and preferentially bound AnV (Chapter 3). Furthermore, monoclonal antibodies to CD14, CD66b, CD62P, TF and PSGL-1 were titrated to establish the appropriate concentration to identify MP expressing these surface markers. Following optimisation, flow cytometry was used to enumerate total MP and MP bearing CD14, CD66b and TF, as well as triple-stained TF+MP with either CD14 or CD66b (Chapter 4). In Chapter 5, MP bearing CD62P and PSGL-1 were identified and co-stained with TF.

BS patients not only had significantly greater total, CD14+ and TF+MP counts compared with HC, but these were greater in BS patients with a history of thrombosis compared to those without (all $p < 0.001$). CD66b+ MP were also increased in BS and Th+ patients compared with HC and Th- Patients, respectively ($p < 0.001$ and < 0.01). This was the same for CD62P+ and PSGL-1+ MP co-staining with TF in BS and Th+ patients, compared with HC and Th- patients, respectively (both $p < 0.001$).

Based on this co-staining, TF+ MP are derived in part from cells of monocyte and neutrophil lineage. The release of TF+MP from monocytes and/or neutrophils provides a possible link between inflammation and thrombosis in BS. It remains controversial as to whether TF+ MP derive from platelets [631, 632, 634], and endothelial cells express TF *in vitro*, but it is again controversial whether these cells

express TF *in vivo* [214, 215]. Both activated platelets and endothelial cells express CD62P.

TF+ MP have been shown to facilitate clot propagation in preclinical models [597, 649], and have been associated with thrombosis in sepsis, malignancy and venous thromboembolism [212, 318, 332]. As AnV+ MP and MP bearing TF have been shown to have procoagulant activity, coagulation lysis, calibrated automated thrombin generation and factor Xa generation assays were run to investigate this further in Chapter 6.

Coagulation lysis assays were run with reteplase as the lytic agent. Although no difference was shown between BS patients and HC, or between Th+ and Th- patients, MP had a shorter $Tlag_c$ than autologous PPP and MPPP. It has been shown that MP adhere to a biomaterial surface by phosphatidylserine, and may expose TF thereby promoting coagulation [577]. It was shown that blocking of both contact and TF-dependent pathways led to an increase in $Tlag_c$ because of MP (Figure 6-6).

In the CAT assay, there was a significantly increased peak thrombin in BS and Th+ patients than HC and Th- patients, respectively. There was, however, no correlation between MP count and calibrated thrombin generation assay measures. Factor Xa generation by MP was measured by a proprietary capture ELISA against a standard curve of positive TF controls, so that a calculation of TF concentration in samples could be made. This showed that HC had a higher level of TF compared to BS patients, whilst Th+ TF concentration was higher than Th-. These results, however, were within the “normal range”.

It was unclear why there was a discrepancy between the MP and coagulation assay results, especially that of the FXa generation assay. I investigated whether there

was a significant presence of TFPI bearing MP, which might counteract TF function. There was significantly raised TFPI + MP in BS *versus* HC, in count and proportion (both $p < 0.001$), whilst TFPI+ MP was significantly increased in proportion, but not count, in Th- than in Th+ patients ($p < 0.001$). The TFPI+ MP/TF+ MP (TFPI/TF ratio) was identified as having a possible association with BS and thrombotic BS. There were no BS patients with a history of thrombosis who had a TFPI/TF ratio > 0.7. It was thought that this ratio may help to identify those at risk of thrombosis in BS ($p < 0.001$)

8.2 Flow cytometry and assessment of microparticles

The haemostasis and extracellular vesicle research communities are endeavouring to standardise flow cytometry analysis of MP [216, 257, 271]. Efforts at standardisation have focussed on all aspects of flow cytometry of MP, from pre-analytical to analytical aspects [216, 218]. This has resulted in several different protocols utilised by research teams, so direct comparison between studies is complex.

There are several strengths to using flow cytometry. It remains the most commonly used technique in the literature to assess MP, since they can be enumerated and their cellular source identified by surface binding of fluorochrome-conjugated antibodies. In this thesis, there were known limitations of the technique for quantitation of MP, but there is no definitive method for quantitation, so the current method was employed. This method consistently showed significantly increased MP count in BS and Th+ patients, compared to HC and Th- patients, respectively. This is the first time any MP measure has identified with a phenotype of BS. Previous

study of CD62P+ MP by flow cytometry did not establish this difference [601], making this association novel.

There are a few issues with the technique chosen, commencing with pre-analytical issues. I used the protocol as described by the Brogan group [203, 343, 344, 584, 587]. This has been published several times, so I did not evaluate different sample preparation protocols utilised by other groups [216, 218]. This could be considered a weakness of the study, especially since, as per the work of Brogan and colleagues, I added a high-speed centrifugation step to isolate MP from PPP prior to analysis.

The concerns with the extra centrifugation step are MP aggregates and loss of MP number. In this study, the final centrifugation step was 17,000g for 60min. One group has shown that there is an 80% decline in MP when centrifuging PPP at 13,000g for 2min, which is often used in the haemostasis community to analyse MP [216, 249], whilst CD31+ CD42b- MP declined significantly with a centrifugation step of 10,000g for 10min [227]. The concern is that multiple centrifugation steps leads to a loss of MP from the sample, especially the smaller MP. Many flow cytometers, however, are unable to detect MP < 500nm in diameter, so this loss may not impact on MP count [268, 271]. Also, this is much less than the loss that occurs with ultracentrifugation speeds that are used by other groups. There are considerable issues with ultracentrifugation, such as aggregation and shearing effects [257].

Although it is a weakness not to have investigated other centrifugation techniques, this technique was consistently applied to all samples in this thesis and a difference was noted, as with other studies [203, 343, 344, 584, 587]. The choice was also pragmatic, because it allowed processing of a reasonably substantial number of samples and to obtain data on cellular origin and other surface markers. Also, it is known that not all MP are able to be detected by flow cytometry, so detecting a selection consistently is no less valid.

Swarming is when smaller MP coalesce as microaggregates, and are measured as one rather than multiple single events [280]. This could be increased with the high-speed centrifugation step used in this thesis, which may have led to increased microaggregates. In the analytical step, the initial 'boost' utilised by flow cytometers and the event rate can considerably affect this [218, 281, 282]. The greater the boost and event rate, the more likely there will be aggregates and swarming, so both parameters were kept shorter and lower, respectively, to avoid this. I did not, however, confirm whether there was an issue with swarming, which could have been achieved by electron microscopy, nanoparticle tracking analysis and Imagestream flow cytometry. The latter has been shown to image particles as small as 100nm in diameter, so may have imaged microaggregates [279].

MP were isolated from PPP, because I used fluorochrome-conjugated AnV as the surface marker. During MP release, there is rearrangement of the lipid bilayer, such that there is preferential expression of phosphatidylserine on the surface of MP [185]. AnV requires a calcium-containing buffer to bind surface phosphatidylserine, so if used with PPP there would be clotting.

There are alternative membrane stains that could have been used. Biomaleimide binds cysteine and thiol groups in cell membrane and does not require an additional washing step [275]. Apart from avoiding a high-speed centrifugation step, it is often utilised by groups who wish to identify all MP, including the AnV- group [276]. With flow cytometry, gating can be set to identify AnV- MP when using AnV as the main cell surface marker [273]. The main issue with this technique, and using membrane dyes other than AnV, is misidentifying lipoproteins as MP, especially if participants have not fasted [218]. This is the same when not using a cell surface marker. Groups using no cell surface markers are often dependent on using latex sizing beads to identify MP. There are differences in the refractive index of MP compared

to latex beads, so precise sizing of MP is difficult [271, 280]. Also, in this thesis, for practical reasons, participants were not fasting, so analysis of AnV- negative MP, may have led to false elevated results, as would using an alternate membrane dye or no cell surface marker.

The consistency in MP counts was demonstrated in MP running pooled PPP samples (Figure 3-9). This was not, however, demonstrated longitudinally with multiple samples from individual patients. This would have been ideal to confirm reproducibility.

8.3 Cohort of Behçet's Syndrome patients

Peripheral blood samples were collected from 88 BS patients who presented to dedicated clinics at St. Thomas' Healthcare and Imperial College Healthcare NHS Trusts. This is a large cohort of BS patients, with 21 thrombotic BS (Th+) patients, which is not out of keeping with the frequency of thrombotic patients in non-endemic regions [600]. This however, may be slightly increased compared to other groups in Turkey and the Middle East. Often a thrombotic episode is the trigger for referral to the clinic, which may account for the increased representation of BS patients with a thrombotic phenotype in this cohort.

As described in Chapter 4, the increased frequency of pulmonary emboli in this cohort was possibly due to a misdiagnosis, such as an inflamed thrombus in the pulmonary artery, and it can be difficult to distinguish between the two [518, 567]. We were unable to confirm this, since there was no access to the original imaging, such as CT pulmonary angiography or ventilation perfusion scans.

In this cohort, there was a heterogeneous population of BS patients and this was a retrospective analysis. Many of the patients were referred from other centres, so the

accuracy of documentation of initial onset of symptoms, thrombotic episode or other manifestation of BS was variable. Therefore, in many of the cases, it was not possible to give a time between the thrombotic episode and sampling, although most were greater than a year. Also, many of the patients were on immunomodulatory therapy, including just over a third on prednisolone (median dose 8.75 mg daily). Studies have shown that treatment of inflammatory conditions leads to a drop in MP counts [605]. It may well be that treatment has lowered the MP count in this cohort of BS patients and the differences in absolute MP counts and MP proportions between untreated BS patients and healthy controls are actually greater. Despite this, differences in MP count and proportion were upheld in comparison with the HC group, and between Th+ and Th- groups.

The retrospective nature of this study is a limitation. A longitudinal study would have allowed multiple sampling of the same patients to see if there was any variability over time and establish that there was reproducibility in the BS cohort. It is also possible that the differences identified, especially between those BS patients with and without thrombosis, may have followed rather than preceded the thrombotic episode. This, however, is unlikely considering that for most of the patients the thrombotic episode preceded blood sampling by greater than a year.

It would have been ideal to perform a prospective study to see if MP, or the TFPI/TF ratio, identified BS patients with thrombosis. Such a study, however, would be complex to perform. This cohort of BS was recruited over a couple of decades, so the frequency of presentation is low, the trigger for referral is often thrombosis and many of the patients are established on anticoagulation and/or immunomodulatory therapy prior to review [568, 600]. Although this was a relatively large study of BS in the UK, substantiating these observations in BS patients from endemic areas in Turkey, the Middle East and Korea would be important to validate the findings.

It is not clear from the findings whether they are specific to BS or representative of a general process that occurs in inflammatory and thrombotic conditions. If there were comparator inflammatory and thrombotic populations, then it could be seen whether they are due to BS or is part of a systemic process that occurs in these conditions. It is possible that the findings are not specific to BS, but are a pathological response to inflammation and thrombosis. Raised MP and TF+ MP have been seen in septicaemia [666], rheumatoid arthritis [336, 338, 604], SLE [273, 284] and malignancy [315]. This BS cohort had a BSDAI at median 2/12 [571]. Although this measure is not used clinically and remains a research tool, this represents a very low disease activity and, clinically, the patients were stable. Other surrogate markers for inflammation, such as CRP and ESR, are often normal or modestly elevated in BS, whilst current routinely used coagulation studies are not useful [400].

8.4 Functional work

Coagulation-lysis, CAT and Zymuphen MP-TF assays were done to assess the coagulant function BS MP. The coagulation lysis assay was performed without the use of exogenous TF, and with reteplase as the lytic agent. Although, there was no difference found between BS patient and HC groups, nor with Th+ and Th- groups, there was one consistent finding. The MP provided a shorter $Tlag_c$ then autologous PPP. The plasma used with MP was autologous MPPP, and in this context, adhered MP led to a shortening of the $Tlag_c$. To confirm the presence of MP, the well surface could have been scraped and MP resuspended and put through a flow cytometer or undergone nanoparticle tracking analysis.

Mackman's group tend to assess MP not by flow cytometry or imaging, but by in-house coagulation assays, such as thrombin antithrombin or FXa generation assays [306, 307, 667]. From this, it could be assumed that since adhered MP were assessed in autologous MPPP, the shortening $Tlag_c$ and prolonging $Tlag_l$ confirms the presence of MP in these wells. Moreover, MP are known to adhere to the well service, and been shown to promote coagulation in a TF-dependent manner [577].

In this thesis, the increased coagulant activity was through a combination of the contact and TF-dependent pathways, since only a blockade of both led to a rise in $Tlag_c$ (Figure 6-6). This was only run once and would require repetition, to confirm its veracity. There was only one concentration of both CTI and anti-TF neutralising antibody used. It is possible by varying the concentration of these that the extent of contribution of both pathways could be ascertained. Also, the impact of AnV was not investigated. AnV has been shown to abrogate thrombin generation by MP from childhood patients with active vasculitis, as assessed by the CAT assay [343].

It may have been possible to examine the clot after the initiation of the coagulation phase thereby not adding reteplase to the system. Hess showed that impaired fibrinolysis in diabetes was due to increased complement incorporation into clots, and that clots had thinner but tighter packed fibrin strands [668]. This was shown by confocal microscopy and it would have been useful to see if clots formed by BS patients had similar characteristics, especially in those with the thrombotic phenotype.

The CAT and Zymuphen MP-TF assays did not demonstrate the expected differences. The CAT assay showed a rise in peak thrombin in BS and Th+ patients, compared with HC and Th- patients respectively. This difference was only found when using a five-fold increased concentration of MP in pooled normal MPPP, showing this assay to be relatively insensitive. This is in keeping with many previous

studies that have not found a clotting assay that identifies BS patients at risk of thrombosis. In keeping with this, there was no correlation found between any MP count and the parameters of the CAT assay.

The Zymuphen MP-TF assay showed that the amount of TF in BS patients was less than in HC, although both values were in normal range at < 2pg/ml ($p < 0.001$; Figure 6-13). There was no difference found between BS patients with and without thrombosis. In combination with the CAT assay, this suggests there are other factors reducing thrombin and FXa generation. Immunostaining has shown that TF is often latent rather than active, and activates in areas of the vessel wall where there is vascular inflammation and MP are trapped by fibrin or in neutrophil extracellular traps [597].

In the Zymuphen MP-TF assay, PPP rather than an MP suspension was used. It is possible that there was an increased circulation of fibrinolytic factors that would impair TF function. A reactive increase in TFPI has been seen in BS patients with a thrombotic tendency [660]. It would have been useful to check the TFPI level by ELISA of BS and HC PPP, and use TFPI blocking antibodies. It has been shown that TFPI blocking antibodies increases the sensitivity of thrombin generation assays [90]. The other possibility is that there was not enough MP in the sample. In many of the studies that show increased thrombin generation, samples came from participants with malignancy or sepsis, so there is a either a high concentration of tumour-derived TF+MP or a persistent inflammatory stimulus for cellular release of TF+ MP [315, 633, 669]. In this BS population, the participants had relatively inactive disease.

8.5 TFPI

The variable presence of TFPI may explain the discrepancy between MP count and coagulation assay results [90]. The finding that TFPI+ MP were increased in BS compared with HC is consistent with a previous report showing raised total plasma TFPI by ELISA [660]. This was explained as a reactive response that may reduce the risk of thrombosis in this setting. The finding that the proportion of TFPI+ MP was significantly lower in Th+ compared with Th- patients, however, goes against this. It is unclear which cell-derived MP carry TFPI, and whether they are the same as those that carry TF. Nevertheless, the data suggest that the balance between TF and TFPI determines thrombotic risk. Low TFPI is known to lead to increased activity of the TF-dependent pathway, and an increased incidence of thrombosis in the general population [73, 89].

TFPI is also able to downregulate TF expression on monocytes [670] and reduced TFPI+ MP has been associated with increased resistance to thrombolysis in acute myocardial infarctions and increased risk of gestational vascular complications in pregnancy [652, 655]. By downregulating TF expression on monocytes, then there would be a reduction in the release of TF+ MP from activated monocytes.

The TFPI/TF ratio > 0.7 was shown to be associated with a decreased risk of thrombosis, since all BS patients with a history of thrombosis in this cohort had a TFPI/TF ratio < 0.7. This is an association and is not a marker of a disease activity. It would need to be validated in other larger cohorts of BS and in prospective longitudinal studies, and so, for that reason, this cut-off may not remain the same. The other aspect of this cut-off is that there are several patients without thrombosis who also had a ratio < 0.7. This may mean that the cut-off is simply an association, without any functional significance or that these patients are more likely to have a thrombotic episode in the future.

The reason why there is a difference in the number of TFPI expressing MP in BS patients is unclear. It could include differential synthesis of TF and TFPI by activated monocytes [88], variable TFPI degradation by leucocyte-derive serine proteases and reactive oxygen species [661, 662] and the influence of genetic levels on TFPI levels [664].

8.6 Future work

To establish the value of the outcomes of this study, the following further clinical work is planned:

- To compare BS with inflammatory and thrombotic control groups
- To substantiate findings in a larger cohort from Turkey or elsewhere
- To perform a prospective study to assess the value of MP levels and TFPI/TF ratio in predicting first thrombosis
- To assess the impact of biologics on MP level and TFPI/TF ratio

There were several weaknesses in this study, with the most prominent being the lack of inflammatory and thrombotic comparison cohorts. This would help to establish whether the findings are disease-specific. MP have been shown to be increased in rheumatoid arthritis [283, 335, 336], SLE [284, 671-673] and connective tissue disorders [340, 341]. Suppression of inflammation has been shown to cause a decline in endothelial cell derived MP in SLE [605]. This would suggest that inflammation has a significant impact.

Venous thrombosis from any cause has been shown to be associated with an increased level of MP [181, 201, 332, 651, 674]. It may be that thrombosis and inflammation are directly associated with MP release, particularly TF+ MP, and it is

not a finding that is specific to BS. This is possibly the case, but the TFPI/TF ratio has not been assessed in either situation.

This BS cohort is relatively large for the UK, and other non-endemic regions, but it would be important to substantiate the observations in larger groups from endemic regions with more patients, such as Turkey, the Middle East, Korea and Japan. This was a retrospective analysis, so a prospective longitudinal study would lead to more information on the natural history of MP in these patients. This would be difficult to do in the UK, since it is such a rare condition.

Many of the patients were referred by another centre, once they had developed an organ-specific manifestation of BS, leading to anticoagulation and immunomodulatory therapy being given prior to being seen. This would impact on studies, and make comparisons difficult in the UK [568, 600].

It was unclear whether there was a significant impact from biologics, since some of the participants were also on anti-TNF therapy. It would be useful to see whether biologics impacted on the level of MP and TFPI/TF ratio. Additionally, assessing baseline MP count and TFPI/TF ratio in untreated patients to see whether this measure can identify those will develop a thrombosis.

8.7 Conclusion

This thesis shows that MP, especially TF+ MP, were increased in BS patients, and are particularly associated with the thrombotic phenotype. The novel finding that all Th+ patients had a TFPI/TF ratio < 0.7, suggests that the TFPI/TF imbalance towards TF may be associated with thrombosis. This remains an observation and it is too early to suggest this is a biomarker for thrombotic risk in BS, until it is studied in validation cohort, such as from Turkey, the Middle East or Korea. Furthermore,

prospective longitudinal studies are required to see if the findings represent a response to the thrombotic event or precede it. This has not been confirmed in this thesis. Be that as it may, these findings are associated with the thrombotic phenotype of BS and may act as a platform for further research in an understudied and poorly understood clinical group that remains a management challenge.

9 References

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Appendix 1

Behçet's Syndrome Manifestations

ID	Age	M/F	Mouth Ulcers	Genital Ulcers	Pustules / Acne	EN	Eye – drops	Eye - systemic	Poor healing	Pathergy	Headaches	Fatigue	Neurological problems	GI problems	GI ulcers on colonoscopy	Joint Pain	Joint swelling	Aneurysm	BSDAI
BS11	26	F	2	1	2	2	0	0	2	2	1	2	0	2	0	2	0	0	3
BS13	48	M	1	0	1	1	0	0	0	0	2	1	1	0	0	0	0	0	0
BS2	61	F	2	2	2	1	0	0	2	2	0	2	0	2	0	2	0	0	4
BS8	33	M	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	3
BS16	45	F	1	1	1	1	1	2	0	2	2	1	1	2	0	1	1	0	2
BS1	39	M	2	1	1	1	0	0	2	0	0	0	0	0	0	0	0	0	2
BS3	56	M	2	2	1	0	0	0	1	1	2	1	0	0	0	1	0	0	3
BS4	23	F	2	2	0	0	0	0	0	0	0	2	0	0	0	2	0	0	1
BS5	44	F	2	0	0	0	0	0	1	1	1	2	0	0	0	2	0	0	2
BS6	43	F	1	1	0	0	1	1	0	0	2	2	0	0	1	1	0	0	1
BS7	43	F	2	2	1	1	0	0	2	2	2	2	0	0	0	2	0	0	3
BS9	48	M	2	1	0	0	1	1	0	0	0	0	0	1	0	1	1	0	1
BS10	26	M	1	1	0	0	0	0	1	0	1	1	1	0	0	1	0	0	0
BS12	37	F	2	1	2	0	1	0	0	0	0	2	0	0	0	0	0	0	2
BS14	26	M	2	1	2	0	0	0	2	1	2	2	2	0	0	2	0	0	4
BS15	40	F	2	1	1	2	0	0	1	1	2	2	0	0	0	2	0	0	3
BS51	64	F	1	2	2	0	0	0	1	1	1	2	0	1	0	1	0	0	2
BS17	32	M	2	1	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1
BS18	58	M	1	1	1	0	1	0	1	0	0	2	0	1	0	2	1	0	0
BS19	23	F	1	1	0	0	0	0	0	2	2	1	0	0	0	2	0	0	0
BS20	18	M	1	1	1	2	0	0	1	1	2	2	2	0	0	2	1	0	0
BS21	67	F	2	1	1	0	1	1	0	2	1	2	0	1	0	2	1	0	1

ID	Age	M/F	Mouth Ulcers	Genital Ulcers	Pustules / Acne	EN	Eye – drops	Eye - systemic	Poor healing	Pathergy	Headaches	Fatigue	Neurological problems	GI problems	GI ulcers on colonoscopy	Joint Pain	Joint swelling	Aneurysm	BSDAI
BS22	58	F	2	1	2	1	1	1	1	1	2	2	0	2	0	1	1	0	1
BS23	37	M	2	1	0	0	0	0	0	0	0	2	0	2	0	2	0	0	2
BS24	46	F	1	0	0	0	1	1	2	0	1	2	0	0	0	2	1	0	0
BS25	50	M	2	0	1	0	0	0	1	1	2	2	1	0	0	0	0	0	1
BS26	31	M	2	2	2	0	0	0	0	0	0	2	0	0	0	2	1	0	3
BS27	46	F	1	0	2	0	0	0	0	2	2	2	0	2	0	2	0	0	4
BS28	45	F	1	0	2	0	2	2	2	2	2	2	0	2	0	2	0	0	1
BS29	44	M	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	0	0
BS30	47	M	2	1	2	2	0	0	1	0	1	1	0	2	0	2	1	1	6
BS31	59	F	1	1	1	1	1	1	1	1	2	2	0	1	0	2	1	0	6
BS32	27	M	2	2	2	0	0	0	0	0	2	2	0	2	0	2	2	0	3
BS33	34	M	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	1	0
BS35	37	M	1	1	2	1	0	0	0	1	0	1	0	0	0	1	0	1	1
BS36	33	M	2	1	1	0	1	1	0	0	2	2	1	1	0	2	0	0	3
BS37	26	F	2	1	1	1	0	0	1	1	1	1	0	1	0	1	0	0	3
BS38	64	F	2	2	0	1	0	1	1	0	2	2	0	2	0	2	1	1	5
BS39	17	F	2	1	1	0	0	0	1	1	1	0	1	1	1	0	0	0	0
BS40	59	F	2	2	1	1	2	2	2	1	2	2	2	2	0	2	1	0	4
BS41	58	F	2	1	2	1	1	0	1	1	2	2	0	2	2	2	2	0	6
BS42	27	F	1	1	1	1	0	0	1	1	1	1	0	1	1	1	0	0	0
BS43	33	M	2	1	0	0	1	1	0	1	2	2	1	1	0	1	0	0	1
BS44	25	M	2	1	1	0	1	1	0	0	0	1	1	0	0	2	0	0	1
BS45	28	M	2	0	1	0	0	0	1	1	0	0	0	0	0	1	1	0	0
BS46	27	M	1	1	1	0	1	0	0	0	1	1	1	0	0	1	0	0	0
BS47	59	F	2	1	0	0	1	0	2	1	1	2	0	0	0	1	0	0	0
BS48	48	F	2	1	2	0	0	0	2	2	1	2	0	1	0	2	2	0	4
BS49	58	F	2	0	0	0	0	0	2	2	2	2	0	2	0	2	0	0	2

ID	Age	M/F	Mouth Ulcers	Genital Ulcers	Pustules / Acne	EN	Eye – drops	Eye - systemic	Poor healing	Pathergy	Headaches	Fatigue	Neurological problems	GI problems	GI ulcers on colonoscopy	Joint Pain	Joint swelling	Aneurysm	BDAI
BS50	42	F	1	0	1	0	2	2	2	1	1	2	2	2	0	2	1	0	3
BS53	45	M	1	2	0	1	1	0	0	0	0	2	2	2	0	1	0	0	4
BS54	37	M	2	2	2	0	0	0	2	2	1	1	0	0	0	1	0	1	0
BS55	33	M	1	1	0	0	1	1	0	0	1	1	1	1	0	1	0	0	0
BS56	27	F	1	1	1	1	0	0	1	1	1	1	0	1	0	1	0	0	0
BS57	46	F	2	0	2	2	1	2	1	1	2	2	0	2	0	2	2	0	4
BS58	48	F	2	2	2	0	0	0	2	2	2	2	1	2	2	2	2	0	7
BS59	42	F	2	1	1	0	0	0	2	2	2	2	0	2	0	1	0	0	0
BS60	58	F	2	1	2	1	1	0	1	1	2	2	0	1	1	1	1	0	6
BS61	60	F	2	2	2	0	0	0	2	2	1	2	0	1	0	1	0	0	3
BS62	64	F	1	2	2	1	1	0	2	0	2	2	0	2	0	2	0	0	4
BS63	36	M	2	1	1	1	0	1	0	1	1	1	0	1	0	0	0	0	1
BS64	49	M	1	1	2	2	0	0	1	1	1	2	0	1	0	2	2	0	5
BS65	48	M	1	1	2	0	1	1	2	0	1	1	0	2	0	2	2	0	0
BS66	44	F	1	1	1	0	1	1	2	2	1	1	0	0	1	1	1	1	0
BS67	39	F	2	2	2	0	0	0	0	1	1	1	0	0	1	0	0	0	2
BS68	48	F	2	1	2	0	0	0	2	2	1	2	0	1	0	2	2	0	5
BS69	36	M	2	0	2	0	1	2	0	0	0	0	0	0	0	2	2	0	3
BS70	43	M	1	1	2	0	1	2	2	0	2	2	0	2	0	2	0	0	4
BS71	46	M	1	1	1	0	1	2	0	0	2	2	0	1	0	2	0	0	2
BS72	16	M	2	0	1	0	0	0	1	1	2	2	0	0	0	2	0	2	4
BS73	74	M	1	0	1	1	1	1	0	0	1	2	0	0	0	2	0	0	1
BS74	51	M	2	1	2	0	1	2	1	0	1	1	0	0	0	2	0	0	3
BS75	26	M	2	0	2	0	1	2	0	0	2	2	0	2	0	0	0	0	1
BS76	42	M	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1
BS77	58	M	1	1	1	0	1	2	0	0	0	0	0	0	0	2	0	0	2
BS78	42	F	1	1	2	0	1	1	0	0	2	2	0	1	0	2	2	0	2

ID	Age	M/F	Mouth Ulcers	Genital Ulcers	Pustules / Acne	EN	Eye – drops	Eye - systemic	Poor healing	Pathergy	Headaches	Fatigue	Neurological problems	GI problems	GI ulcers on colonoscopy	Joint Pain	Joint swelling	Aneurysm	BDAI
BS79	37	F	1	1	1	0	1	2	0	0	0	2	0	0	0	0	0	0	0
BS80	36	M	1	2	2	0	1	1	2	2	0	2	0	2	0	1	1	0	2
BS81	43	F	1	1	2	0	0	2	2	2	2	2	0	0	0	2	1	0	3
BS82	52	M	1	1	1	0	1	1	1	0	1	2	0	2	0	2	0	0	2
BS83	42	F	2	1	1	2	1	2	2	2	2	2	0	0	0	1	0	0	3
BS84	31	M	1	0	2	0	1	1	0	0	1	1	0	0	0	1	0	0	2
BS85	58	M	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
BS86	44	M	1	1	2	0	1	2	2	2	2	2	0	2	0	2	0	0	4
BS87	44	M	1	0	0	1	2	2	0	0	1	0	0	0	0	2	0	0	1
BS88	35	M	2	0	0	0	2	2	0	0	2	2	2	0	0	1	0	0	2
BS89	44	F	1	1	2	0	1	2	2	2	1	2	0	0	0	2	1	0	2
BS90	32	M	1	1	2	1	0	0	0	2	2	2	0	0	0	0	0	0	0

KEY – 0 = never present; 1 = previously present, currently quiescent; 2 = currently present

Healthy Controls

ID	Age	Gender	Other Co-morbidity	Thrombosis history	Smoker
HC21	24	F	Nil	nil	Never
HC23	44	M	Nil	nil	Never
HC22	32	F	Mouth ulcers	nil	Never
HC24	47	F	Mouth ulcers	nil	Never
HC25	33	M	Nil	nil	Never
HC26	41	M	Nil	Nil	Never
HC27	35	F	Nil	Nil	Never
HC28	36	M	Nil	Nil	Never
HC29	35	F	Nil	Nil	Never
HC30	24	F	Nil	Nil	Never
HC31	31	M	Nil	Nil	Never
HC32	34	M	Nil	Nil	Never
HC33	34	F	Nil	Nil	Never
HC34	37	M	Nil	Nil	Never
HC35	41	M	Nil	Nil	Never
HC36	29	M	Nil	Nil	Never
HC37	41	M	Nil	Nil	Never
HC38	31	M	Nil	Nil	Never
HC39	24	M	Nil	Nil	Never
HC40	22	M	Nil	Nil	Never
HC41	24	F	Nil	Nil	Never
HC42	37	M	Nil	Nil	Ex-smoker
HC43	18	M	Nil	Nil	Never
HC44	35	F	Nil	Nil	Never
HC45	37	M	Nil	Nil	Ex-smoker
HC59	34	F	Nil	Nil	Never
HC60	35	F	Nil	Nil	Never
HC61	37	M	Nil	Nil	Never
HC62	49	F	Nil	Nil	Never
HC63	29	M	Nil	Nil	Never
HC64	25	F	Nil	Nil	Never

ID	Age	Gender	Other Co-morbidity	Thrombosis history	Smoker
HC65	39	M	Nil	Nil	Never
HC66	35	M	Nil	Nil	Never
HC67	53	F	Nil	Nil	Never
HC68	37	F	Nil	Nil	Never
HC69	40	M	Nil	Nil	Never
HC70	32	F	Nil	Nil	Never
HC71	25	M	Nil	Nil	Never
HC72	36	F	Nil	Nil	Never
HC73	37	M	Nil	Nil	Never
HC74	28	F	Nil	Nil	Never
HC75	43	F	Nil	Nil	Never
HC76	58	F	Nil	Nil	Ex-smoker
HC77	50	F	Nil	Nil	Current
HC78	52	F	Nil	Nil	Never
HC79	53	F	Nil	Nil	Never
HC80	30	F	Nil	Nil	Never
HC81	42	M	Nil	Nil	Never
HC82	42	F	Nil	Nil	Never
HC83	45	M	Nil	Nil	Never
HC84	43	M	Nil	Nil	Never
HC85	66	F	Nil	Nil	Never
HC86	40	M	Nil	Nil	Never
HC87	47	M	Nil	Nil	Never
HC88	37	M	Nil	Nil	Never
HC89	49	F	Nil	Nil	Never
HC90	42	M	Nil	Nil	Never
HC91	29	M	Nil	Nil	Never
HC92	28	M	Nil	Nil	Never
HC93	31	M	Nil	Nil	Never
HC94	57	M	Nil	Nil	Never
HC95	64	F	Nil	Nil	Never
HC96	68	M	Nil	Nil	Never
HC97	65	M	Nil	Nil	Never

ID	Age	Gender	Other Co-morbidity	Thrombosis history	Smoker
HC98	33	M	Nil	Nil	Never
HC99	26	F	Nil	Nil	Never
HC100	37	M	Nil	Nil	Never
HC101	41	M	Nil	Nil	Never

All Patients – Thrombotic History and Cardiac risk factors

ID	LL DVT	PE	Other VTE	FHx VTE	Anti-coagulated	Aspirin	Diabetes	CVS	HT	Atopy	CVS Meds	Comments
BS11	0	0	0	0	0	0	0	0	0	0		
BS13	1	0	0	0	1	0	0	0	0	1		
BS2	0	0	0	0	0	0	0	0	0	1		
BS8	1	1	0	0	2	0	0	0	1	0		
BS16	0	0	0	0	0	0	0	0	0	0		
BS1	1	0	0	0	2	0	0	0	1	0		
BS3	0	0	0	0	0	0	0	0	2	0		
BS4	0	0	0	0	0	0	0	0	0	0		
BS5	0	0	0	0	0	0	0	0	0	0		
BS6	0	0	0	0	0	1	0	0	0	0		
BS7	2	0	0	0	0	0	0	0	0	0		
BS9	0	0	0	0	0	0	0	0	0	0		
BS10	0	0	0	0	0	0	0	0	0	0		
BS12	0	0	0	0	0	0	0	0	0	0		
BS14	0	0	2	0	2	0	0	0	0	0		
BS15	1	0	0	0	0	1	0	0	0	0		
BS51	0	0	0	0	0	0	0	0	2	0		
BS17	0	0	1	0	2	0	0	0	0	0	Simvastatin	Smoker
BS18	0	0	0	0	0	0	0	0	0	0		Ex-smoker
BS19	0	0	0	0	0	0	0	0	0	1		
BS20	0	0	0	0	0	0	0	0	1	0	Bendrofluazide	
BS21	0	0	0	0	0	0	0	0	0	0		
BS22	0	0	0	0	0	0	0	0	0	0		Smoker
BS23	0	0	0	0	0	0	0	0	0	0		
BS24	0	0	0	0	0	0	0	0	2	0	Nifedipine	Smoker

ID	LL DVT	PE	Other VTE	FHx VTE	Anti-coagulated	Aspirin	Diabetes	CVS	HT	Atopy	CVS Meds	Comments
BS25	0	1	1	0	2	0	0	0	0	0		
BS26	0	0	1	0	0	0	0	0	0	0		Smoker
BS27	1	0	1	0	2	0	0	0	0	0		Smoker
BS28	0	0	0	0	0	0	0	0	2	0	Nifedipine, frusemide	
BS29	1	0	0	0	1	0	0	0	0	0		Ex-smoker
BS30	0	0	0	0	0	0	0	0	0	0		
BS31	0	0	0	0	0	0	0	0	0	0		
BS32	0	0	0	0	0	0	0	0	2	0		
BS33	1	1	0	0	0	0	0	0	0	0		
BS35	0	0	0	0	0	1	0	0	0	0		
BS36	0	0	0	0	0	0	0	0	0	0		
BS37	0	0	0	0	0	0	0	0	0	0		
BS38	0	0	0	0	0	0	0	0	2	0	Amlodipine	
BS39	0	0	0	0	0	0	0	0	0	0		
BS40	0	0	0	0	0	0	0	0	0	0		
BS41	0	0	0	0	0	0	0	0	0	0		
BS42	0	0	0	0	0	0	0	0	0	1		
BS43	0	0	0	0	0	0	0	0	0	0		
BS44	0	0	0	0	0	0	0	0	0	0		
BS45	1	0	0	0	2	0	0	0	0	0		Smoker
BS46	0	0	0	0	0	0	0	0	0	0		
BS47	0	0	0	0	0	0	0	0	0	0		
BS48	0	0	0	0	0	2	0	0	0	0		
BS49	0	0	0	0	0	0	0	0	0	0		
BS50	0	0	0	0	0	0	0	0	0	2		
BS53	0	0	0	0	0	0	0	0	0	0		
BS54	0	0	0	0	0	1	0	0	0	0		Smoker
BS55	0	0	0	0	0	0	0	0	0	0		

ID	LL DVT	PE	Other VTE	FHx VTE	Anti-coagulated	Aspirin	Diabetes	CVS	HT	Atopy	CVS Meds	Comments
BS56	0	0	0	0	0	0	0	0	0	0		
BS57	0	0	0	2	0	2	2	0	2	0		
BS58	0	0	0	0	0	0	0	0	0	0	Frusemide	Smoker
BS59	0	0	0	2	0	0	0	0	0	0		Smoker
BS60	0	0	0	0	0	0	0	0	0	0		
BS61	0	0	0	0	0	0	0	0	0	0		
BS62	0	0	0	0	0	0	0	0	1	0		
BS63	1	0	0	0	2	0	0	0	0	0		Smoker
BS64	0	0	0	0	0	0	0	0	0	0		
BS65	0	0	0	0	0	0	0	0	2	0		Ex-smoker
BS66	0	0	0	0	0	0	0	0	0	0		
BS67	0	0	0	2	0	0	0	0	0	0		Ex-smoker
BS68	0	0	0	0	0	0	0	0	0	0		
BS69	0	0	0	0	0	0	0	0	0	0		Ex-smoker
BS70	0	0	1	0	0	2	0	2	2	0	Bisoprolol, perindopril, amlodipine, atorvastatin	Smoker
BS71	0	0	1	0	0	0	0	0	2	0	Amlodipine, atenolol, olmesartan	Ex-smoker
BS72	1	1	1	0	2	0	0	0	0	0		
BS73	0	0	1	0	0	0	0	0	0	0		Ex-smoker
BS74	1	0	1	0	0	0	0	0	0	0		Ex-smoker
BS75	0	0	0	1	0	0	0	0	0	0		
BS76	0	0	1	0	1	2	0	0	2	0	Amlodipine	
BS77	2	0	1	0	2	0	0	0	0	0		
BS78	0	0	1	0	0	0	0	0	0	0		Smoker
BS79	0	0	0	0	0	0	0	0	0	0		
BS80	0	0	0	0	0	0	0	0	0	0		Ex-smoker

ID	LL DVT	PE	Other VTE	FHx VTE	Anti-coagulated	Aspirin	Diabetes	CVS	HT	Atopy	CVS Meds	Comments
BS81	0	0	0	0	0	0	0	0	0	0		Smoker
BS82	1	0	1	1	2	0	0	0	0	0		Smoker
BS83	1	0	1	2	2	0	0	0	0	0		
BS84	0	0	0	0	0	0	0	0	0	0		
BS85	0	0	1	0	0	0	0	0	2	0	Bendrofluazide	Smoker
BS86	0	0	0	0	0	2	0	1	2	0	Bendrofluazide, ISMN, sildenafil, rosuvastatin, bisoprolol, perindopril	Smoker
BS87	1	0	0	0	2	0	0	0	0	0		Ex-smoker
BS88	0	0	0	0	0	0	0	0	0	0		
BS89	0	0	0	0	0	0	0	0	0	0		Smoker
BS90	0	0	0	0	0	0	0	0	0	0		Smoker

KEY – LL – lower leg; DVT – deep venous thrombosis; PE – pulmonary emboli; FHx – family history; CVS – cardiovascular; HT - hypertension

Listed Treatments of Behçet's Syndrome Patients

ID	Colchicine	Azathioprine	Mycophenloate	Cyclosporin	Methotrexate	Adalimumab	Etanercept	Infliximab	Calcium/vitamin D	Hydroxychloroquine	Leflunomide	Prednisolone	Other
BS11	2	1	2	0	0	0	0	0	0	0	0	0	Pentoxifyline
BS13	1	2	0	0	0	0	0	0	0	0	0	1	Venlafaxine, warfarin
BS2	1	1	0	1	0	0	0	0	2	0	0	1	MST, celecoxib, amitriptyline, paroxetine, pregabalin
BS8	1	2	0	0	0	0	0	0	1	0	0	1	Warfarin, amitriptyline, baclofen, DHC
BS16	0	0	0	0	2	0	0	2	2	0	0	2	
BS1	2	0	0	0	0	0	0	0	0	0	0	0	warfarin
BS3	0	2	0	0	0	0	0	0	2	0	0	2	Thalidomide, dapsone, pizotifen, bendrofluazide
BS4	2	0	0	0	0	0	0	0	0	0	0	0	Betnesol, diclofenac, omeprazole
BS5	2	1	0	0	0	0	0	0	0	0	0	0	
BS6	1	1	2	0	0	0	0	2	0	0	0	1	Meloxicam, aspirin, omeprazole, spasmonal
BS7	2	1	0	0	0	0	0	0	0	0	0	0	
BS9	2	0	0	0	0	0	0	0	0	0	0	1	Pentoxifylline
BS10	0	2	0	0	0	0	0	2	0	0	0	1	
BS12	0	0	0	0	0	0	0	0	0	0	0	0	
BS14	0	2	0	0	0	0	0	0	0	0	0	0	
BS15	1	1	0	0	0	0	0	0	0	0	0	0	
BS51	2	0	0	0	0	0	0	0	2	0	0	1	

ID	Colchicine	Azathioprine	Mycophenloate	Cyclosporin	Methotrexate	Adalimumab	Etanercept	Infliximab	Calcium/\vitamin D	Hydroxychloroquine	Leflunomide	Prednisolone	Other
BS17	0	1	2	0	0	0	0	0	0	0	0	1	Omeprazole
BS18	0	2	0	0	0	0	0	2	1	0	0	2	Omeprazole
BS19	1	0	0	0	0	0	0	0	0	0	0	2	
BS20	1	2	0	0	0	0	0	0	2	0	0	2	Ptzotifen, testosterone
BS21	2	0	0	0	0	0	0	0	2	0	0	0	Citalopram, acyclovir, propranolol, HRT
BS22	1	1	1	2	0	0	0	0	2	0	0	2	Risedronate, omeprazole, buscopan, amitriptyline
BS23	2	0	0	0	0	0	0	0	0	0	0	0	
BS24	0	1	1	1	0	0	0	0	2	0	0	2	Alendronate, omeprazole, paroxetine
BS25	0	0	0	0	2	0	0	0	2	0	0	2	Amitriptyline, iron tabs, alendronate, celecoxib, paracetamol
BS26	2	0	0	0	0	0	0	0	0	0	0	0	
BS27	0	0	0	0	0	0	0	0	0	0	0	1	
BS28	0	1	2	0	0	0	0	0	1	0	0	2	Tacrolimus
BS29	2	0	0	0	0	0	0	0	0	0	0	0	
BS30	0	1	0	0	2	0	0	2	2	0	0	2	Lansoprazole, folic acid, aledronic acid
BS31	1	1	1	0	0	1	0	0	0	0	0	0	
BS32	0	1	0	0	0	2	0	0	2	1	0	1	
BS33	1	2	0	0	0	0	0	0	1	0	0	1	Warfarin, amitriptyline
BS35	0	1	2	0	0	0	0	1	1	0	0	2	
BS36	0	0	1	1	2	0	0	2	2	0	0	1	

ID	Colchicine	Azathioprine	Mycophenloate	Cyclosporin	Methotrexate	Adalimumab	Etanercept	Infliximab	Calcium/\vitamin D	Hydroxychloroquine	Leflunomide	Prednisolone	Other
BS37	2	1	1	0	0	0	0	0	0	0	0	0	Pentoxyfiline
BS38	2	0	0	0	0	0	0	0	2	0	0	1	
BS39	0	0	0	0	0	0	0	0	0	0	0	0	
BS42	1	1	1	0	0	0	0	0	0	0	0	0	Pentoxifylline
BS43	0	0	1	1	2	0	0	2	2	0	0	1	
BS44	0	2	0	0	0	0	0	0	0	0	0	1	
BS45	0	0	0	0	0	0	0	0	0	0	0	2	warfarin
BS46	0	2	0	0	0	0	0	2	0	0	0	1	
BS47	2	0	0	0	0	0	0	0	0	0	0	0	
BS48	0	0	0	0	2	0	0	0	2	0	0	2	
BS49	0	1	0	0	0	0	0	0	0	0	0	1	
BS50	0	0	0	0	0	0	0	0	0	2	0	0	antihistamines
BS53	0	1	2	1	0	0	0	1	2	0	0	1	
BS54	0	1	2	0	0	0	0	1	0	0	0	1	
BS55	0	0	1	1	2	0	0	2	2	0	0	1	
BS56	2	1	1	0	0	0	0	0	1	0	0	0	
BS57	0	1	2	0	0	2	0	0	2	0	0	2	
BS58	1	2	0	0	1	2	1	1	0	0	0	2	
BS59	0	1	1	0	0	0	0	0	2	0	0	2	
BS60	1	1	1	0	0	0	0	0	1	0	0	2	
BS61	0	1	1	0	0	0	2	0	0	0	0	0	Previously – sulphasalazine and thalidomide
BS62	2	0	0	0	0	0	0	0	2	0	0	1	
BS63	2	0	0	0	0	0	0	0	0	0	0	0	

ID	Colchicine	Azathioprine	Mycophenloate	Cyclosporin	Methotrexate	Adalimumab	Etanercept	Infliximab	Calcium/\vitamin D	Hydroxychloroquine	Leflunomide	Prednisolone	Other
BS64	1	1	2	0	0	0	0	0	0	0	0	2	
BS65	1	0	0	2	0	0	0	0	2	0	0	2	
BS66	1	1	2	1	1	2	0	1	2	0	0	2	
BS67	1	0	0	0	0	0	0	0	0	0	0	0	
BS68	0	0	0	0	2	0	0	0	2	0	0	2	
BS69	2	2	0	0	0	0	0	0	0	0	0	1	
BS70	1	1	2	1	0	0	0	0	2	0	0	1	Amitriptyline, lansoprazole
BS71	2	2	0	0	0	0	0	0	2	0	0	1	Doxycycline, pregabalin, sertaline
BS72	0	0	0	0	0	0	0	2	0	0	0	1	Pregabalin, tramadol, paracetamol
BS73	2	1	0	0	0	0	0	0	0	0	0	2	Codydramol, etidronate, diclofenac gel
BS74	0	2	0	0	0	0	0	0	2	0	0	1	Alendronate, omeprazole
BS75	2	0	0	0	0	0	0	0	2	0	0	1	Citalopram, timolol, lopidine eye drops, lumagen
BS76	2	1	0	0	0	0	0	0	1	2	0	1	Citalopram
BS77	0	1	1	1	0	0	0	0	2	0	0	2	
BS78	2	1	2	0	0	0	0	0	2	0	0	2	Ranitidine, alendronate, betamethasone mouthwash
BS79	2	2	0	0	1	0	0	0	2	0	0	1	

ID	Colchicine	Azathioprine	Mycophenloate	Cyclosporin	Methotrexate	Adalimumab	Etanercept	Infliximab	Calcium/\vitamin D	Hydroxychloroquine	Leflunomide	Prednisolone	Other
BS80	2	2	0	2	0	0	0	0	2	0	0	1	Etoricoxib, ibuprofen, paracetamol
BS81	1	1	0	0	0	0	0	0	0	0	0	2	6-Mercaptopurine, venlafaxine
BS82	1	2	0	0	0	0	0	0	2	0	0	1	warfarin
BS83	1	1	1	0	0	0	0	0	2	0	0	2	Omeprazole, zoladex, enoxaparin, anastrazole,
BS84	1	1	0	0	0	0	0	0	0	0	0	1	
BS85	0	1	0	1	0	0	0	0	0	0	0	1	
BS86	2	0	2	1	0	0	0	0	2	0	0	2	Previously IFN-α, doxycycline, ibuprofen, sildenafil
BS87	2	2	0	0	0	0	0	0	2	0	0	2	
BS88	0	0	0	0	0	0	0	0	0	0	0	2	Aciclovir, omeprazole, Maxidex
BS89	1	1	0	0	0	0	0	0	1	0	0	2	6-Mercaptopurine
BS90	2	1	0	0	0	0	0	0	2	0	0	1	Betamethasone mouth wash

Appendix 2

Correlation Between CAT Assay Measures and MP Counts

Endogenous Thrombin Potential (ETP)

Category	All	BS	HC
Total	r = 0.08882 p = 0.7729	r = 0.2871 p = 0.0619	r = 0.4160 p = 0.0764
TF+	r = 0.2137 p = 0.4832	r = 0.1633 p = 0.2953	r = 0.1852 p = 0.4477
CD14+	r = 0.2197 p = 0.4708	r = 0.1695 p = 0.2772	r = 0.1419 p = 0.5623
TF+/CD14+	r = 0.2159 p = 0.4786	r = 0.1531 p = 0.3270	r = 0.1169 p = 0.6336

Peak Thrombin (PT)

Category	All	BS	HC
Total	r = 0.1047 p = 0.7217	r = 0.3206 p = 0.017	r = 0.1268 p = 0.4750
TF+	r = 0.1959 p = 0.5022	r = 0.2032 p = 0.1368	r = 0.1173 p = 0.5090
CD14+	r = 0.1980 p = 0.4975	r = 0.2075 p = 0.1286	r = 0.3255 p = 0.0603
TF+/CD14+	r = 0.1880 p = 0.5197	r = 0.1875 p = 0.1703	r = 0.02352 p = 0.8949

Time to Thrombin Generation ($Tlag_c$)

Category	All	BS	HC
Total	r = -0.1395 p = 0.6342	r = -0.1549 p = 0.2635	r = 0.0461 p = 0.7991
TF+	r = -0.2980 p = 0.3007	r = -0.1393 p = 0.3149	r = -0.0203 p = 0.9106
CD14+	r = -0.2959 p = 0.3043	r = -0.1351 p= 0.3302	r = 0.0796 p = 0.6596
TF+/CD14+	r = -0.2728 p = 0.3454	r = -0.1266 p = 0.3615	r = 0.0548 p = 0.7619

Appendix 3

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A low balance between microparticles expressing tissue factor pathway inhibitor and tissue factor is associated with thrombosis in Behçet's Syndrome

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Thrombosis is common in Behçet's Syndrome (BS), and there is a need for better biomarkers for risk assessment. As microparticles expressing Tissue Factor (TF) can contribute to thrombosis in preclinical models, we investigated whether plasma microparticles expressing Tissue Factor (TF) are increased in BS. We compared blood plasma from 72 healthy controls with that from 88 BS patients (21 with a history of thrombosis (Th+) and 67 without (Th−). Using flow cytometry, we found that the total plasma MP numbers were increased in BS compared to HC, as were MPs expressing TF and Tissue Factor Pathway Inhibitor (TFPI) (all $p < 0.0001$). Amongst BS patients, the Th+ group had increased total and TF positive MP numbers (both $p \leq 0.0002$) compared to the Th- group, but had a lower proportion of TFPI positive MPs ($p < 0.05$). Consequently, the ratio of TFPI positive to TF positive MP counts (TFPI/TF) was significantly lower in Th+ *versus* Th− BS patients ($p = 0.0002$), and no patient with a TFPI/TF MP ratio >0.7 had a history of clinical thrombosis. We conclude that TF-expressing MP are increased in BS and that an imbalance between microparticulate TF and TFPI may predispose to thrombosis.

Behçet's syndrome (BS) is a multisystem inflammatory disorder, characterized by oral and genital ulceration, skin lesions, arthritis and uveitis[1]. Venous thrombosis occurs in 10–40% of cases, and is a leading cause of morbidity and mortality in the condition[2–4]. Venous thrombosis particularly occurs in young males with a history of inflammatory eye disease, and is most severe in this group. Immunomodulation is considered to be central to the management of BS patients with thrombotic complications[5], but the role of anticoagulation is still controversial[6].

The cause of thrombosis in Behçet's syndrome remains unknown. A number of studies have looked for changes in the hemostatic and fibrinolytic systems in BS, but no clear abnormality has been adopted for measurement in clinical practice[7]. Consequently, there is a need both for better understanding of the pathophysiology and for biomarkers to aid in clinical management.

Tissue Factor (TF, CD142) is a 47 kDa transmembrane cell surface glycoprotein that triggers the extrinsic coagulation pathway[8]. It is highly expressed by stromal cells within the adventitia of blood vessels, providing a protective 'haemostatic envelope' that activates blood coagulation upon vascular injury. However, TF also has a major pathologic role in linking thrombosis with inflammation[9]. On the one hand TF expression is induced by inflammatory activation of monocytes/macrophages by endotoxin or inflammatory cytokines[10,11], and on the other hand the generation of thrombin via TF expression leads to the inflammatory activation of cells via protease-activated receptors[12].

The importance of circulating microparticles (MP) to disease is increasingly appreciated[13]. Although MP are highly heterogeneous, a population derived from cell plasma membranes can be identified by annexin V binding to surface phosphatidylserine (PS). Circulating annexin V+ MP expressing TF (TF+MP) harbor the majority

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	Healthy Controls		Behçet's Syndrome	
General Demographics				
Cohort size	74		88	
Median age (range)	41	(18–75)	43	(17–74)
Male sex (%)	40	54%	47	53%
Current smoker	6	8%	12	14%
Clinical Characteristics				
Mucocutaneous	—		88	100%
Erythema nodosum	—		29	33%
Pathergy/poor wound healing	—		48	55%
Musculoskeletal - arthralgias	—		71	81%
Arthritis	—		30	34%
Vascular	—		22	25%
Aneurysm	—		7	8%
Thrombosis	—		21	24%
Posterior/panuveitis	—		39	44%
Gastrointestinal inflammation	—		10	11%
Neurological	—		18	20%
Median BSDAI*	—		2	
Current medications				
Colchicine	—		22	29%
Azathioprine	—		15	19%
Mycophenolate	—		12	16%
Anti-TNF therapy	—		16	21%
Cyclosporine	—		3	4%
Methotrexate	—		9	12%
Steroids†	—		24 (7.5 mg)	31%
Warfarin	—		12	14%

Table 1. Details of patients and controls. *Behçet's Syndrome Disease Activity Index = BSDAI. †Median dose in brackets.

of TF in blood plasma and have been considered to be primarily derived from monocyte/macrophage membranes[14,15]. Not only have TF+ MP been found to contribute to clot propagation in preclinical models[16–18], but numbers of circulating TF+ MP have been related to clinical thrombosis in sepsis, atherosclerosis, malignancy and venous thromboembolism[19–22].

Tissue Factor Pathway Inhibitor (TFPI) is a single chain Kunitz-type proteinase inhibitor that is primarily produced by endothelial cells, although it is also synthesized by other cell types including monocytes[23,24]. TFPI acts as an anti-coagulant through inhibiting Factor Xa, and, once bound to Factor Xa, inhibits the Factor VIIa/TF complex[25,26]. The majority of TFPI in the circulation is the truncated beta form, linked to the surface of vascular endothelium by a glycophosphatidylinositol anchor[27]. The full length alpha form also binds endothelial cells, probably via glycosaminoglycans, and circulates in plasma bound to lipoproteins[28]. However TFPI may be also detected on some circulating MP[29,30]. Low overall plasma levels of TFPI have been associated with recurrent venous thrombosis in the general population[31]. A previous study observed increased levels of plasma TFPI in BS patients overall, although a difference between those with and without a history of thrombosis was not addressed[32]. The amount of TFPI that can be mobilized into the circulation by heparin infusion has been found to be reduced in BS, suggesting that TFPI associated with endothelium is reduced and raising the possibility that this might confer a prothrombotic risk[33].

Quantities of plasma MP are known to be increased in BS, but previous studies these have not addressed an association between MP and thrombosis[34,35]. We investigated the hypothesis that numbers of plasma MP expressing TF are increased in BS. We found that this is the case, and that patients with a low ratio of MP counts expressing TFPI to MP counts expressing TF are significantly more likely to have a history of thrombosis.

Results

Study population. The demographic and clinical details of the 88 BS patients and the 74 age- and sex-matched HC in the study are shown in Table 1. Current BS disease activity was low (median BSDAI score 2 out of a maximum of 12) with no difference between those with (Th+) or without (Th−) a history of thrombosis (Fig. 1). Within the BS group, 21 (24%) had a history of thrombosis, including 13 lower limb deep vein thromboses, 11 retinal vein occlusions (8 branch retinal vein and 3 central retinal vein), 2 superior vena cava occlusions and 1 dural sinus thrombosis. Six patients had had more than one thrombotic episode. Three patients had been told that they had suffered a pulmonary embolus, two of whom had a previous deep vein thrombosis and one a dural sinus thrombosis.

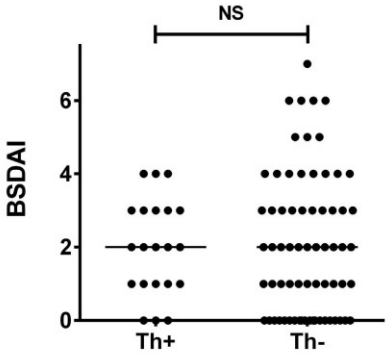


Figure 1. Scoring on the Behçet's Syndrome Disease Activity Index (BSDAI) of BS patients in the study, comparing patients with (Th+) and without (Th−) a history of thrombosis. There was no significant difference between the two groups.

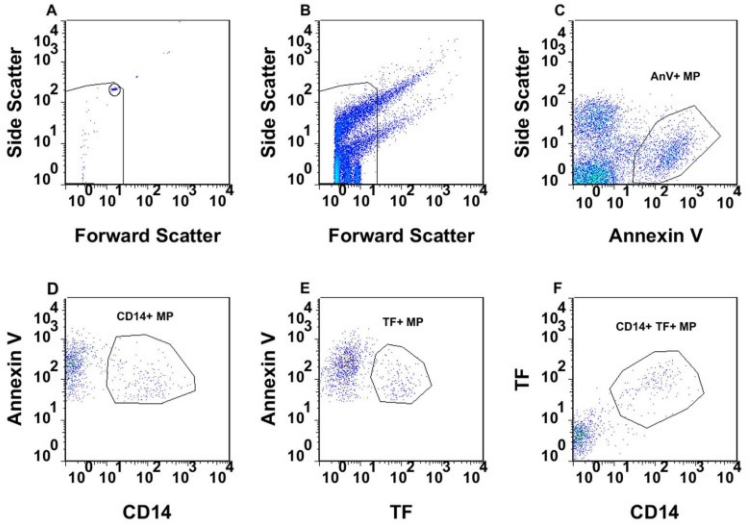


Figure 2. Gating strategy for flow cytometry. All MP were gated $<1.1\,\mu m$ in size using microbeads. **(A,B)** Forward and side scatter of **(A)** $1.1\,\mu m$ microbead suspension and **(B)** plasma microparticles ; **(C)** particles $<1.1\,\mu m$ binding annexin V (AnV+) were defined as MP; **(D,E)** gating of annexin V positive MP positive for **(D)** CD14 or **(E)** TF; **(F)** annexin V positive MP stained by both anti-CD14 and anti-TF.

Quantification of annexin V positive MP. We defined and enumerated plasma MP using flow cytometry by gating on particle size ($<1.1\,\mu m$), reactivity with annexin V and with specific antibodies (Fig. 2, Supplementary Figure 1). As shown in Fig. 3A, BS patients had an increased absolute total MP count compared to HC (medians: 6.18×10^5/ml versus 1.90×10^5/ml; $p < 0.0001$). By staining with antibodies, we found that the number of CD14 positive MP (CD14+ MP) was also increased, either when expressed as absolute number of CD14+ MP (medians: 1.17×10^5/ml versus 2.05×10^4/ml; $p < 0.0001$; Fig. 3B), or as a percentage of total MP (medians: 16.97% versus 9.18%; $p < 0.0001$; Fig. 3C). This suggests that the increased MP in BS derive at least in part from monocytes and monocyte-derived macrophages, although neutrophils also express CD14 at low

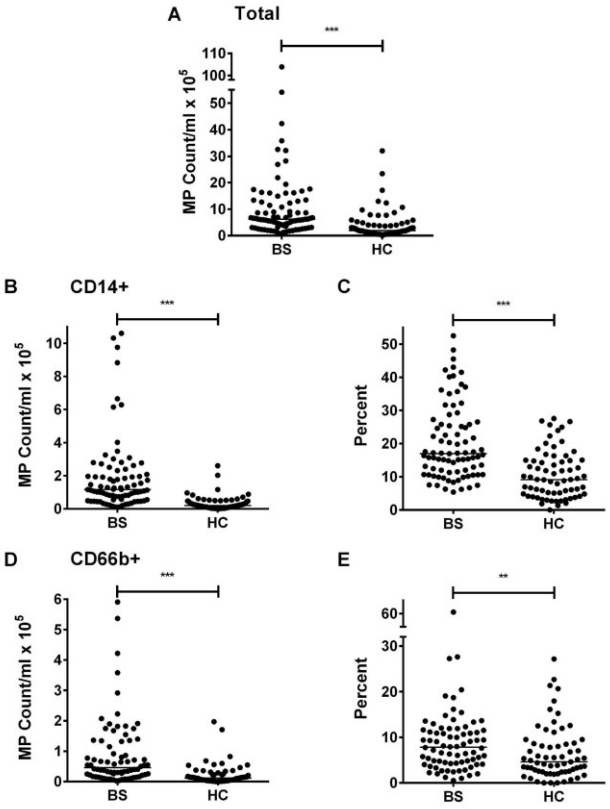


Figure 3. Behçet's Syndrome (BS) patients have higher MP counts than HC. MP were enumerated in BS *versus* HC plasma by flow cytometry using annexinV and mAb against CD14 and CD66b. **(A)** total MP; **(B,C)** CD1+ MP; **(D,E)** CD66b+ MP. Data are expressed as absolute counts **(A,B,D)** or % total MP **(C,E)**. **$p < 0.01$; ***$p < 0.001$.

density and may release TF+ MP[36,37]. An increase in granulocyte-derived MP in BS was indeed suggested by an increase in the absolute number ($p < 0.0001$; Fig. 3D) and percentage ($p < 0.01$; Fig. 3E) of CD66b+ MP. The total of CD14+ and CD66b+ MP did not account for all the MP, but the origins of the remainder were not analysed.

Quantification of MP expressing Tissue Factor. By costaining with anti-TF monoclonal antibody (Fig. 2E), we determined whether numbers of TF+ MP are also increased in BS. This was the case, with both an increase in absolute number (medians: 5.31×10^4/ml versus 6.70×10^3/ml; $p < 0.0001$; Fig. 4A) of TF+ MP and the percentage of total MP that were TF positive (medians: 8.72% versus 3.66%; $p < 0.0001$; Fig. 4B). By triple staining using anti-CD14 (Fig. 4C,D) or CD66b (Fig. 4E,F), we were able to confirm that the increase in TF+ MP in BS patients related at least in part to MP release by cells of the monocytic and/or granulocyte lineage. Differences between BS patients and HC remained after smokers in the BS patient group had been excluded (Supplementary Figure 2). Sub-analysis showed no correlation between MP numbers and increasing age (Supplementary Figure 3). Furthermore, sub-analysis of the BS group did not reveal an effect of corticosteroid treatment on MP numbers (Supplementary Figure 4). Further sub-analyses to relate MP numbers to other individual drugs did not reveal any associations.

Sub-analysis of MP in BS patients according to history of thrombosis. Compared to BS patients without thrombosis (Th−), BS patients with a history of thrombosis (Th+) had a significantly increased absolute number of MP ($p < 0.0001$; Fig. 5A), as well as increased TF+, CD14/TF+, and CD66b/TF+ MP counts

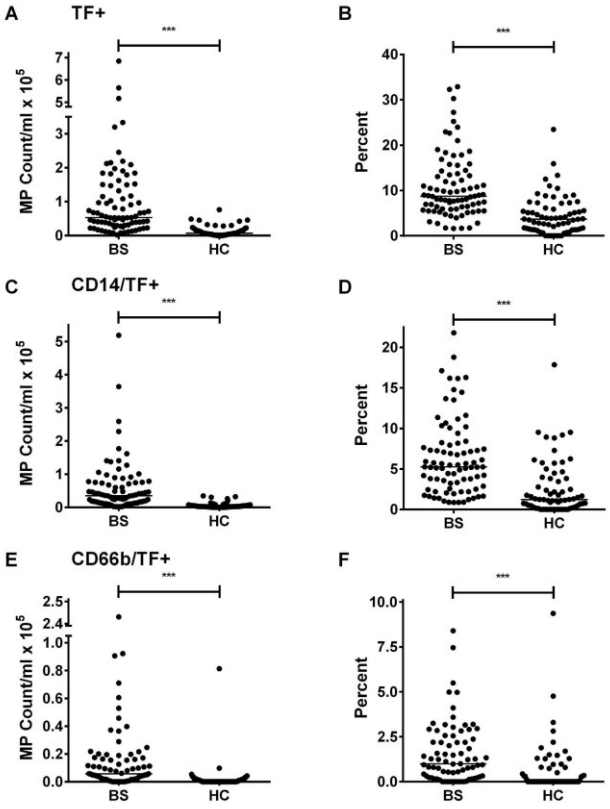


Figure 4. Behçet's Syndrome (BS) patients have higher numbers of TF+ MP than HC. MP were enumerated in BS *versus* HC plasma by flow cytometry using annexin V and mAb. **(A,B)** total TF+ MP; **(C,D)** CD14/TF+ MP; **(E,F)** CD66b/TF+ MP. Data are expressed as absolute counts **(A,C,E)** or % total MP **(B,D,F)**. ***$p < 0.001$.

(Fig. 5B,D,E,G) and percentages (Fig. 5C,E,G), MP numbers were found to be increased in male compared to female BS patients, and this was shown to be due to males having a greater number of Th+ patients (Supplementary Figures 5 and 6). Differences between Th+ and Th− BS patients remained after smokers in the Th+ group were excluded (supplementary Figure 7).

Quantification of MP-induced thrombin generation. Using calibrated automated thrombography (CAT), we found that thrombin generation due to PPP was too low for comparisons to be made. We therefore went on to use five-fold concentration of microparticles suspended in a standard MPPP pooled from HC. Despite this, there were 14 BS samples that did not generate thrombin (1 from a Th+ patient and 13 from a Th− patient), and these were not included in the final analysis. We found a significantly increased endogenous thrombin potential (ETP) and peak thrombin generation in the BS group compared to HC ($p < 0.05$ and <0.01, respectively) (Fig. 6A,C), but no difference between groups in lag time to thrombin generation (not shown). Within the BS group, patients with a history of thrombosis had a significantly increased peak thrombin compared to those without (Fig. 6D; $p < 0.05$), but there was no difference between these groups in ETP (Fig. 6B) or lag time (not shown). Comparing samples that did not generate thrombin between groups, there was no significant difference found between Th+ and Th− BS populations (not shown). Using Pearson correlation coefficient, we were unable to demonstrate significant correlations between MP counts and any index of thrombin generation *in vitro* (not shown).

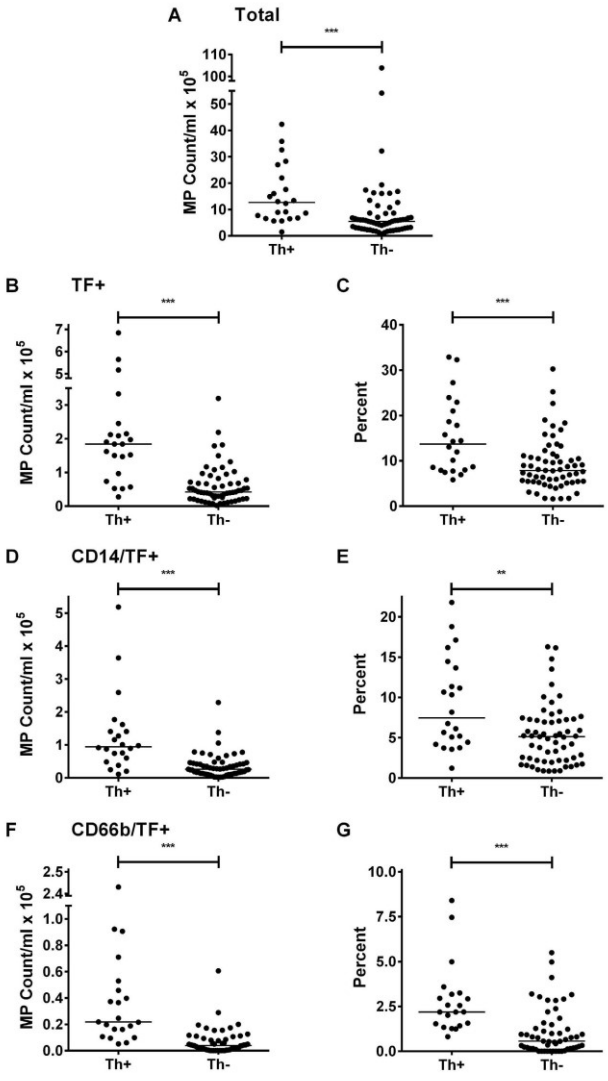


Figure 5. Behçet's Syndrome (BS) patients with a history of thrombosis (Th+) have higher numbers of TF+ MP than those without (Th−). MP were enumerated in Th+ and Th− BS patients by flow cytometry using annexin V and mAb. (**A**) total MP; (**B,C**) total TF+ MP; (**D,E**) CD14/TF+ MP; (**F,G**) CD66b/TF+ MP. Data are expressed as absolute counts (**A,B,D,F**) or % total MP (**C,E,G**). $^{**}p < 0.01$, $^{***}p < 0.001$.

Quantification of MP expressing Tissue Factor Pathway Inhibitor. The failure of the functional assay to show a better relation to TF+ MP numbers might at least in part be explained by variable expression of an inhibitor such as TFPI. As judged by flow cytometry (Supplementary Figure 8), BS patients as a whole had a

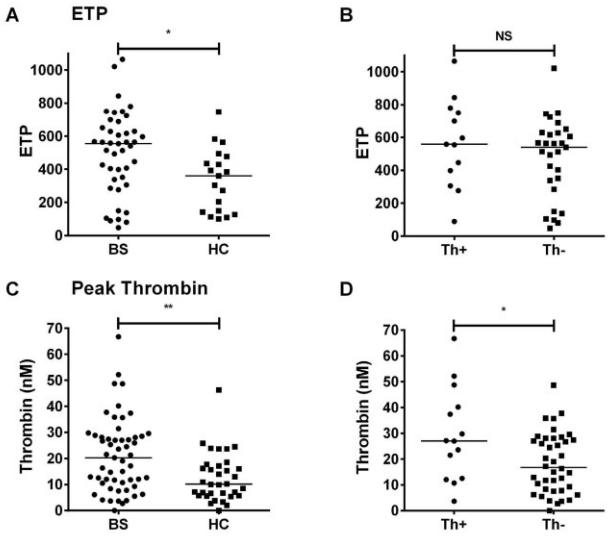


Figure 6. Effects of plasma samples on thrombin generation *in vitro*. Endogenous thrombin potential (ETP) **(A,B)** and peak thrombin **(C,D)** were calculated for (**A,C**) BS compared with HC and (**B,D**) Th+ compared with Th− groups. * $p < 0.05$, ** $p < 0.01$, NS = non-significant.

higher absolute number (medians: 3.60×10^4/ml versus 2.00/ml; $p < 0.0001$; not shown) and percentage (medians: 5.17% versus 0.91%; $p < 0.0001$; Fig. 7A) of TFPI+ MPs, indicating that an increase in TFPI+ MPs in the disease environment may be a protective response. The absolute number of TFPI+ MPs did not significantly differ between Th+ and Th− BS patients (not shown), but, surprisingly, we found that a significantly lower percentage of MP were TFPI+ in the BS patients with a history of thrombosis (Fig. 7B; $p < 0.05$). We calculated the ratio of TFPI+ MPs to TF+ MPs in BS vs HC (Fig. 7C) and Th+ vs Th− (Fig. 7D). BS patients overall had a significantly higher median ratio than HC (0.67 *vs.* 0.25; $p < 0.0001$). Strikingly, Th− BS patients without a history of thrombosis had a higher median TFPI/TF MP ratio compared with Th+ patients (0.72 *vs.* 0.23; $p < 0.0001$), with no patient with a ratio of >0.7 having a history of a thrombotic event.

Discussion

We believe that our data add significantly to the understanding of thrombosis in BS. We found that BS patients not only have significantly higher numbers of MP compared to healthy controls, but also have higher numbers of MP expressing TF. Furthermore, BS patients with a history of thrombosis had higher TF+ MP numbers than those without a thrombosis history. Based on costaining of MP with antibodies against CD14 and CD66, it is likely that TF+ MP derive at least in part from cells of monocyte and/or granulocyte lineage, although other cell types probably also contribute to the circulating TF+ MP pool. The release of TF+ MP by activated monocytes and/or granulocytes provides a plausible link between inflammation and thrombosis in BS.

The population of MP in plasma is highly heterogeneous and there is ongoing debate as to how samples should be prepared and how MP should best be measured[38]. Many laboratories perform a centrifugation step to concentrate MP for analysis[39], and we decided to centrifuge PPP for an hour at 17,000 G, avoiding ultracentrifugation to minimize aggregation. MP were detected by flow cytometry, which is the most common technique used and is more applicable to the clinical setting than others such as atomic force microscopy or nanoparticle tracking analysis. Our decision to use flow cytometry on centrifuged PPP and to focus on annexin V+ MP detectable within a defined forward and side scatter gate set with the use of 1.1 μm latex beads was pragmatic and based on the need to consistently process a reasonably large number of samples and to also obtain data on surface antigens. Two other technical considerations should be noted. First, that we used the unstained MP suspension as the control for annexin V binding, and any background non-specific binding might have been lower by incorporating annexin V in calcium chelating buffer. Secondly, we recognize that differences in the refractive index of MP compared to latex beads may cause difficulties in the precise sizing of the MP we have studied[40].

Our analysis was confined to the Annexin V positive MP population falling within our forward and side scatter gating, and analyzing Annexin V negative MPs in Behçet's syndrome may also be informative[41,42]. However lipoproteins may also fall within the Annexin V negative population[39]. For practical reasons, many of our patients were not fasting, and so we decided not to assess the Annexin V negative data in the present study.

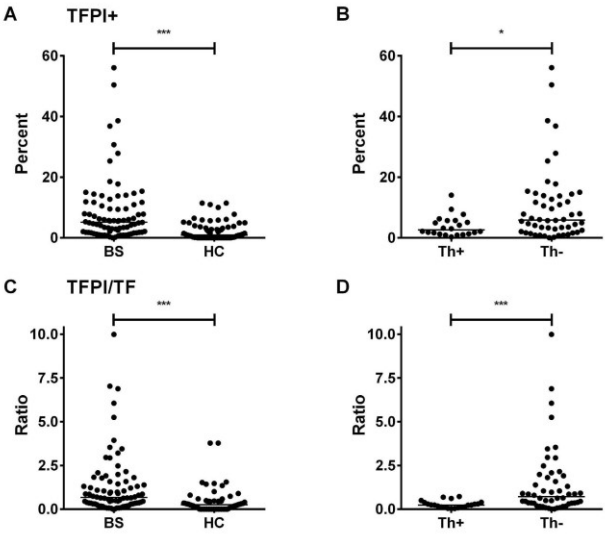


Figure 7. Circulating TFPI+ MP are increased in BS but relatively low in patients with a history of thrombosis. (**A**,**B**) show TFPI+ MP in (**A**) BS *versus* HC and (**B**) Th+ *versus* Th− BS patients expressed as % total MP counts; **(C,D)**; show ratios of TFPI to TF counts in (**C**) BS *versus* HC and (**D**) Th+ *versus* Th− patients.

We initially tested the thrombin generating capacity of BS samples using PPP, but found that the amount of thrombin generated was insufficient to be useful. Consequently, we adapted the assay by testing five-fold concentrates of MP suspended in a standard MPPP that had been pooled from HC. Apart from focusing on the MP fraction, this also enabled us to minimize the effects that any warfarin treatment might have had on coagulation factor concentrations in patient plasma. The results showed that MP-stimulated thrombin generation was relatively poor at differentiating either BS from HC samples or Th+ from Th− samples. This is consistent with many previous studies that have failed to find standard coagulation assays useful in this setting. The lack of correlation between the functional assay and numbers of MP expressing both PS and TF is in keeping with the TF that is recognized on MP by immunostaining being mostly latent rather than active, and it is possible that MP-associated TF only becomes functional in the local milieu of the vessel wall at sites of vascular inflammation once MP are caught by fibrin or neutrophil extracellular traps[17]. The activation of trapped MP-associated TF by disulphide isomerase released locally may then increase the probability of clot propagation and overt thrombosis, as happens in preclinical models[16–18]. A further mechanism enhancing clot propagation in BS may be the recently described oxidative injury to fibrinogen, which makes clots more resistant to lysis[43].

The poor correlation between TF+ MP and the thrombin generation assay may also be explained by the variable presence of TFPI[44]. Our observation that numbers of circulating MP expressing TFPI were significantly increased in BS compared to HC is consistent with a previous report measuring total plasma TFPI by ELISA, and this may be a reactive homeostatic adaptation that serves to reduce the probability of thrombosis in the inflammatory setting[32]. On this background, it was particularly interesting that BS patients with a history of thrombosis had a significantly lower proportion of TFPI+ MP compared to those without. We do not know whether TF and TFPI are expressed by the same or different MPs, but, notwithstanding, the implication of this observation is that the balance between MP-associated TF and TFPI may determine the risk of thrombosis. This is supported by Th− patients having a significantly higher TFPI/TF ratio, and indeed by the absence of a thrombosis history in all individuals with a ratio >0.7. The reasons behind some BS patients having a relatively low level of MP-associated TFPI compared to MP-associated TF are unclear, but could include differential synthesis of TF and TFPI by activated monocytes[24], TFPI degradation by leukocyte-derived serine proteases and/or reactive oxygen species[45,46] and the influence of genetic variants of TFPI on plasma levels[47].

The retrospective nature of our study is an obvious limitation. There is a small possibility that the differences in MP counts that we have detected in the Th− patients followed rather than preceded the thrombotic event. This seems unlikely, as in the majority of patients the interval between thrombosis and MP testing was greater than a year. It would be ideal to conduct a prospective study testing the ability of TF+ and TFPI+ MPs to predict a first thrombosis in BS. However such a project would be difficult to perform, both because thrombosis is often the presenting manifestation, and because treatment has often commenced prior to referral to a specialist centre[48,49]. Although our study is relatively large for a BS study in the UK, it will be important to substantiate the

generalizability of our observations on larger groups from endemic regions with more patients, such as Turkey, the Middle East, Korea and Japan.

The current mainstay of treatment of thrombosis in BS is immunomodulation, either using disease-modifying agents such as azathioprine, or biologic agents such as tumor necrosis factor alpha inhibitors. Anticoagulants alone do not appear to be effective in preventing recurrence. Thus, a small trial in Korea found that thrombosis recurred in three out of four patients treated with anti-coagulation alone, but that immunosuppression, either with or without anticoagulation, was associated with reduced recurrence of thrombosis[50]. Similarly, two larger retrospective studies from France and Turkey have concluded that immunosuppression is associated with reduced thrombosis relapse[49,51].

In conclusion, our study cuts new ground by demonstrating that BS patients may have high circulating levels of MP expressing TF and that these are more numerous in patients with a history of thrombosis. Furthermore, discrimination between patients with and without a history of clinically overt thrombosis was improved by factoring in the level of MP expressing TFPI. The balance between TF+ and TFPI+ MPs may prove useful in thrombosis risk assessment and in making decisions to initiate or withdraw immunosuppressive treatment.

Methods

Ethics. Approval for the study was obtained from the Hammersmith, Queen Charlotte's and Chelsea Hospitals Research Ethics Committee (ref 2001/6179) and all methods were performed in accordance with the relevant guidelines and regulations. All participants gave written informed consent to participate.

Behçet's syndrome patients and healthy controls. This was a cross-sectional study of 88 BS patients and 74 age- and sex-matched healthy controls (HC), all over 18 years old. Patients were recruited from specialist clinics at the Hammersmith and St Thomas' hospitals in London over a period from 2012–2015. The BS patients fulfilled the 1990 International Study Group diagnostic criteria[52]. Specific patient history included BS manifestations, with details of thrombotic episodes. BS disease activity was quantified using the Leeds Behçet's Syndrome Disease Activity Index (BSDAI)[53]. A medication and smoking history was taken from all participants. Healthy controls were free of known disease and not taking medication, and were selected to provide for age and sex-matched comparisons. Laboratory analyses were conducted blind of the identity of plasma donor to avoid bias.

Blood sampling. Peripheral blood was taken from participants using a 21 G needle in accordance to local aseptic non-touch technique guidelines. The first 5 ml were either utilized for serological analyses or were discarded, depending on whether the participant was a BS patient or HC respectively, and 20 mls were transferred into a 50 ml Falcon tube containing 2 ml 3.2% sodium citrate (Sigma, St. Louis, USA).

Platelet poor plasma preparations. Citrated peripheral blood was processed to form platelet poor plasma (PPP) within 1–2 hours of venesection. In the intervening period, care was taken to avoid inverting the sample that could inadvertently lead to the increased production of MP. Samples underwent two 5000 g × 10 min centrifugation steps. After each step, plasma was decanted, being careful not to contaminate it with the pellet. The final PPP preparation was stored as 500 µl aliquots at −80 °C.

MP preparation. Aliquots of PPP were thawed in a 37 °C water bath for 1–2 mins. This was done to prevent the development of cryoprecipitates that would impact on analysis[54]. The aliquots were then centrifuged for 1 hour at 17,000 g at 4 °C to sediment MPs. Subsequently, 480 µl of supernatant was retained as MP poor plasma (MPPP), leaving 20 µl containing the MP pellet. This was resuspended to its original 500 µl volume using Annexin V buffer (BD Pharmingen, Oxford, UK) and stored on ice ready for flow cytometry. For calibrated automated thrombography (CAT), 490 µl of MPPP was retained leaving 10 µl containing the MP pellet ready for use.

Flow cytometry reagents. Reagents used for flow cytometry were: Pacific Blue (PB) conjugated annexin V (Biolegend, San Diego, USA), fluorescein isothiocyanate (FITC) conjugated anti-tissue factor (CD142) (Abd Serotec, clone CLB/TF-5, Oxford, UK), phycoerythrin (PE) conjugated anti-CD14 (Invitrogen, clone Tük4) and PE conjugated CD66b (Biolegend, clone G10F5, San Diego, USA). Murine anti-I K1 monoclonal antibody (Sanquin, clone CLB/TFPI Kunitz domain 1 specific, Amsterdam, Netherlands) was conjugated to AF-647 Apex® molecular beads (Invitrogen Life Sciences, Paisley, UK). The isotype control antibodies were FITC conjugated IgG1 (Abd Serotec, Oxford, UK), PE conjugated IgG1 (Invitrogen, Paisley, UK), PE conjugated IgM_κ (Biolegend, San Diego, USA) and murine IgG1 (Sigma, St. Louis, USA) for binding to AF647 Apex® molecular beads.

Microparticle labelling. The MP suspension (40 µl) was incubated with PB-conjugated annexin V and then co-stained with either PE-labelled anti-CD14 plus FITC-labelled anti-TF, or PE-labelled anti-CD66b plus FITC-labelled anti-TF, or AF-647 conjugated anti-TFPI K1 antibody only. To assist with gating, an unlabeled sample and annexin V buffer was used, as well as the appropriate isotype controls. MPs were incubated at room temperature on a shaker for 30 min in the dark, and the reaction was stopped with the addition on 200 µl annexin V buffer. All flow cytometry reagents were tested and titrated prior to analysis of BS and HC samples. Importantly, PB-annexin V and all conjugated antibodies were initially run in annexin V buffer only to make sure that there was no signal from these reagents, and none was found.

Flow cytometry. A standardized protocol was used to ensure reproducibility and reliable quantification of samples. MPs were identified by forward and side scatter, and by binding of annexin V. Latex beads (1.1 µm diameter; Sigma, St. Louis, USA) were used to confirm MP were below this size, whilst a known concentration of 3 µm latex beads (Sigma, St. Louis, USA) (200,000/ml) was used to enumerate MP during 30 second runs. To

minimize day-to-day flow cytometer variability, this procedure was used before measurement of all samples. Gates setting specific staining with each marker compared to isotype-matched control antibody are illustrated in Supplementary Figure 1. MP counts were calculated using equation 1:

$$MP\ count/ml = \frac{(200{,}000\ \div 3\ \mu m\ bead\ event\ count) \times MP\ event\ count}{Volume\ of\ plasma}$$

Thrombin generation assay. The thrombotic potential of MP was assessed using CAT, which has previously been used to assess the thrombotic potential of MP in a number of conditions[55–57]. To assay PPP, 80 µl with 20 µl buffer were recalcified by addition of $CaCl_2$ and fluorescent substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland), to a final concentration of 16.6 mM and 0.42 mM respectively, in a total volume of 120 µl. To assay MP, 10 µl concentrate of MP was added to 80 µl MPPP (pooled from 10 HC), 10 µl buffer and recalcified by the addition of $CaCl_2$ and fluorescent substrate, as with PPP samples. Thrombin generation from PPP and MP was followed in real time using a Fluoroscan Ascent FL plate reader (Thermo Labsystem, Paisley, UK) and Thrombinoscope software (Synapse BV, Maastricht, Netherlands). Data gathered included: endogenous thrombin potential (ETP), which represents the area under the curve and total thrombin generated; peak thrombin; lag time to thrombin generation. Samples were run in duplicate and only those samples that generated thrombin were included in the analysis.

Statistics. As this was an exploratory study, the numbers of BS patients and healthy controls were arrived at empirically on the basis of availability of BS patients. Results of all the assays followed a non-Gaussian distribution and so are expressed as medians. GraphPad PRISM 6.07 (GraphPad Software, Inc, San Diego, CA, USA) was used for statistical analyses and graph generation. Statistical comparisons of median MP counts, MP proportions and CAT assay measures were accomplished using Mann-Whitney U test with significance set at $p < 0.05$.

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Author Contributions

E.K. was involved in all aspects of the study; N.L.A., A.P.K., M.R.S. and J.C.M. contributed to sample collection; D.E., M.J. and P.B. gave expertise in relation to M.P. flow cytometry; J.A. and M.A.F. gave expertise in relation to thrombin generation assays; D.O.H. contributed to the design of the study and wrote the first draft of the paper; all authors contributed to editing the paper.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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