A study of platelet activation in the antiphospholipid syndrome

Joanne Emily Joseph

MBBS FRACP FRCPA

A thesis submitted to the University of London

for the degree of Doctor of Medicine

University College London, 1999

Abstract

The association between thromboembolism and the presence of antiphospholipid antibodies (APA) is well established, however the mechanism of action of APA in the pathogenesis of thrombosis remains elusive. It is likely that many haemostatic factors are involved.

Platelet activation in antiphospholipid syndrome (APS) has been investigated previously, but many older studies used techniques which were either insensitive or prone to artefactual changes. Whole blood flow cytometry is a sensitive test of platelet activation which allows detection of various aspects of the activation process. The principle aims of this thesis were to use such techniques to investigate both *in vivo* and *in vitro* platelet activation in primary APS (PAPS) and systemic lupus erythematosus (SLE) patients (both with and without secondary APS), and this also necessitated the development of some newer methods. Endothelial cell activation and thrombin generation were also assessed.

Evidence of significantly increased *in vivo* platelet activation (as detected by platelet degranulation, changes in the platelet GPIIb/IIIa complex, and formation of platelet-leukocyte complexes) were found in PAPS, as well as in some cases of SLE. Markers of thrombin generation and endothelial cell activation were also significantly increased in both groups of patients. However, in general these markers did not correlate with each other, suggesting that different processes of activation occur, and this may have been partly due to the effects of different types of therapy (i.e aspirin, warfarin and immunosuppressives). In some assays (particularly the detection of platelet-leukocyte complexes), the changes were more significant in SLE than in

PAPS, suggesting a role of autoantibodies in general. Finally, in vitro experiments failed to demonstrate a platelet-activating effect of APA on normal platelets.

Table of contents

List of tables

List of figures

Abbreviations

5-HT	5-Hydroxytryptamine
ACL	Anticardiolipin antibody
ADP	Adenosine 5'-diphosphate
APA	Antiphospholipid antibody
APC	Activated protein C
APS	Antiphospholipid syndrome
AT	Antithrombin
ATP	Adenosine 5'-triphosphate
β_2GPI	β_2-glycoprotein I
β-TG	β-thromboglobulin
Ca^{++}	Calcium ions
CAPS	Catastrophic antiphospholipid syndrome
CL	Cardiolipin
DG	Diacylglycerol
dRVVT	dilute Russell's Viper venom time
ELISA	Enzyme linked immunosorbent assay
F 1+2	Prothrombin fragment 1.2
FcγRII	Human immunoglobulin G receptor class II
FDP's	Fibrin degradation products
GMP-140	Granule membrane protein-140
GP	Glycoprotein
HCII	Heparin cofactor II
HIT	Heparin induced thrombocytopaenia
HMWK	High-molecular-weight-kininogen
Ig	Immunoglobulin
INR	International normalized ratio
IP_3	Inositol 1,4,5-trisphosphate
KCT	Kaolin clotting time
LA	Lupus anticoagulant
OCS	Open canalicular system

PADGEM	Platelet activation dependent granule-external membrane protein
PAI-1	Plasminogen activator inhibitor-1
PAPS	Primary antiphospholipid syndrome
PDGF	Platelet-derived growth factor
PE	Phosphatidylethanolamine
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PF4	Platelet factor 4
PGI_2	Prostacyclin
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PK	Prekallikrein
PKC	Protein kinase C
PL	Phospholipid
PLA	Platelet-leukocyte aggregate
PLA_2	Phoshpolipase A_2
PLC	Phospholipase C
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PRP	Platelet rich plasma
RGD	Arg-Gly-Asp
SLE	Systemic lupus erythematosus
TAT	Thrombin-antithrombin complex
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
tPA	Tissue plasminogen activator
TSP	Thrombospondin
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
uPA	Urokinase-type plasminogen activator
vWF	von Willebrand factor
WAPS	Warfarin in AntiPhospholipid Syndrome

Publications arising from thesis

Joseph JE, Donohoe S, Harrison P, Mackie IJ, Machin SJ. Platelet activation and turnover in the primary antiphospholipid syndrome. Lupus. 1998; 7: 333-340

Joseph JE, Harrison P, Mackie IJ, Machin SJ. Platelet activation markers and the primary antiphospholipid syndrome. Lupus. 1998; 7: S48-S51

Harrison P, Robinson MSC, Mackie IJ, Joseph J, McDonald SJ, Liesner R, Savidge GF, Pasi J, Machin SJ. Performance of the platelet function analyser PFA-100® in testing abnormalities of primary haemostasis. Blood Coagulation and Fibrinolysis. 1999; 10: 25-31

Joseph JE, Mackie IJ, Harrison P, Machin SJ. Increased thrombin generation in patients with the primary antiphospholipid syndrome. British Journal of Haematology. 1999; 105, S1, 44 (abstract)

Joseph JE, Mackie IJ, Harrison P, Machin SJ. Platelet-leukocyte complexes are increased in patients with systemic lupus erythematosus (SLE) but not primary antiphospholipid syndrome (PAPS). British Journal of Haematology, 1999; 105, S1, 49 (abstract)

Acknowledgements

The work in this thesis was performed during a two year period whilst I was employed as a research registrar at the Haemostasis Research Unit, Unversity College London. I would like to thank my supervisor Professor Samuel J Machin for his support and encouragement during this time, as well as for giving me the opportunity to conduct the project. I am also very grateful to Professor David Isenberg and Dr Hannah Cohen, who allowed me to study their clinic patients. Much thanks goes to Dr Ian Mackie (Senior Lecturer) and Dr Paul Harrison (Lecturer) for teaching me all I needed to know about the practical aspects of studying platelets and antiphospholipid antibodies, as well as providing their guidance and help. I would like to thank all of my colleagues at the Haemostasis Research Unit for their assistance with many of the assays, and their great friendship and support. I am grateful to the staff of the Haematology and Coagulation Departments at University College Hospital for performing the routine assays. Finally I would like to thank the staff of the Haematology and Rheumatology Outpatient Clinics for their assistance in recruiting the many patients for the studies.

1. General Introduction

1.1 Platelets

1.1.1 Production, lifespan and ultrastructure

Platelets are produced from megakaryocytes, which are end-stage nondividing cells derived from proliferating progenitors. Megakaryocytes make up approximately 0.03 to 0.5% of nucleated bone marrow cells (Stenberg & Hill 1998). They appear as large, polyploid cells, and their nucleus contains multiples of the normal 2N complement of DNA. Although the exact number of platelets that a megakaryocyte can produce is unknown, estimates suggest that between 1,000 to 5,000 platelets may be produced per megakaryocyte (Burstein & Breton-Gorius 1995). The precise mechanism of platelet formation and release from the megakaryocyte remains uncertain and two general hypotheses have been proposed (Gewirtz & Schick 1994). The major distinction between them relates to the origin of the megakaryocyte demarcation membrane system (DMS) (which is thought to be formed by invagination of megakaryocyte plasma membrane) and its role in platelet formation. Similarly, the primary site of platelet production is also controversial. One theory proposes that after megakaryocytes mature, they enter the circulation and their cytoplasm fragments into platelets in the pulmonary circulation (Stenberg & Hill 1998). Megakaryocytes have been routinely observed in the microvasculature of the lung (Burstein & Breton-Gorius 1995). On the other hand, since other fully differentiated haematopoietic cells are formed in the bone marrow, many investigators believe that platelets are also likely to be formed and released at this site. The location of megakaryocytes adjacent

to bone marrow sinusoidal lining cells supports this hypothesis (Stenberg & Hill 1998).

The normal range for the platelet count in adults is 150 - 400 $\times$ 10^9/l. Thrombopoiesis is regulated by humoral mechanisms in which the rate of platelet production responds to the number or mass of circulating platelets. Platelet production is largely under the control of thrombopoietin, however other growth factors (such as interleukins-1, 6, and 11; stem cell factor, and granulocyte-colony stimulating factor) are also thought to have some thrombopoietic activity. The bone marrow reserve of platelets is limited and can be rapidly depleted after sudden loss or destruction. Newly formed platelets ('reticulated platelets') contain some rough endoplasmic reticulum and residual mRNA, and are thus able to synthesise small amounts of protein (Kieffer *et al*, 1987). They can be identified as a distinct subpopulation of platelets using supravital dyes and fluorochromes, and their mRNA degrades relatively quickly in the circulation (approximately 24 hours within canine and murine models) (Dale *et al*, 1995; Ault & Knowles 1995). Platelet life span, based on the time required to clear labelled platelets from the circulation, has been estimated to be 8 to 12 days (Stenberg & Hill 1998). Detailed analyses of platelet survival patterns suggest that the majority of platelets survive to senescence while some are randomly removed from the circulation (George & Dale 1995).

Circulating platelets are small, anucleate discoid cells, with a volume of approximately 5 to 8 fl, and a diameter of 1.5 to 3 μm. On blood films they appear as small bluish-grey, oval to round bodies with several purple-red granules. The ultrastructure of a resting platelet is represented in *Figure 1.1*, and its

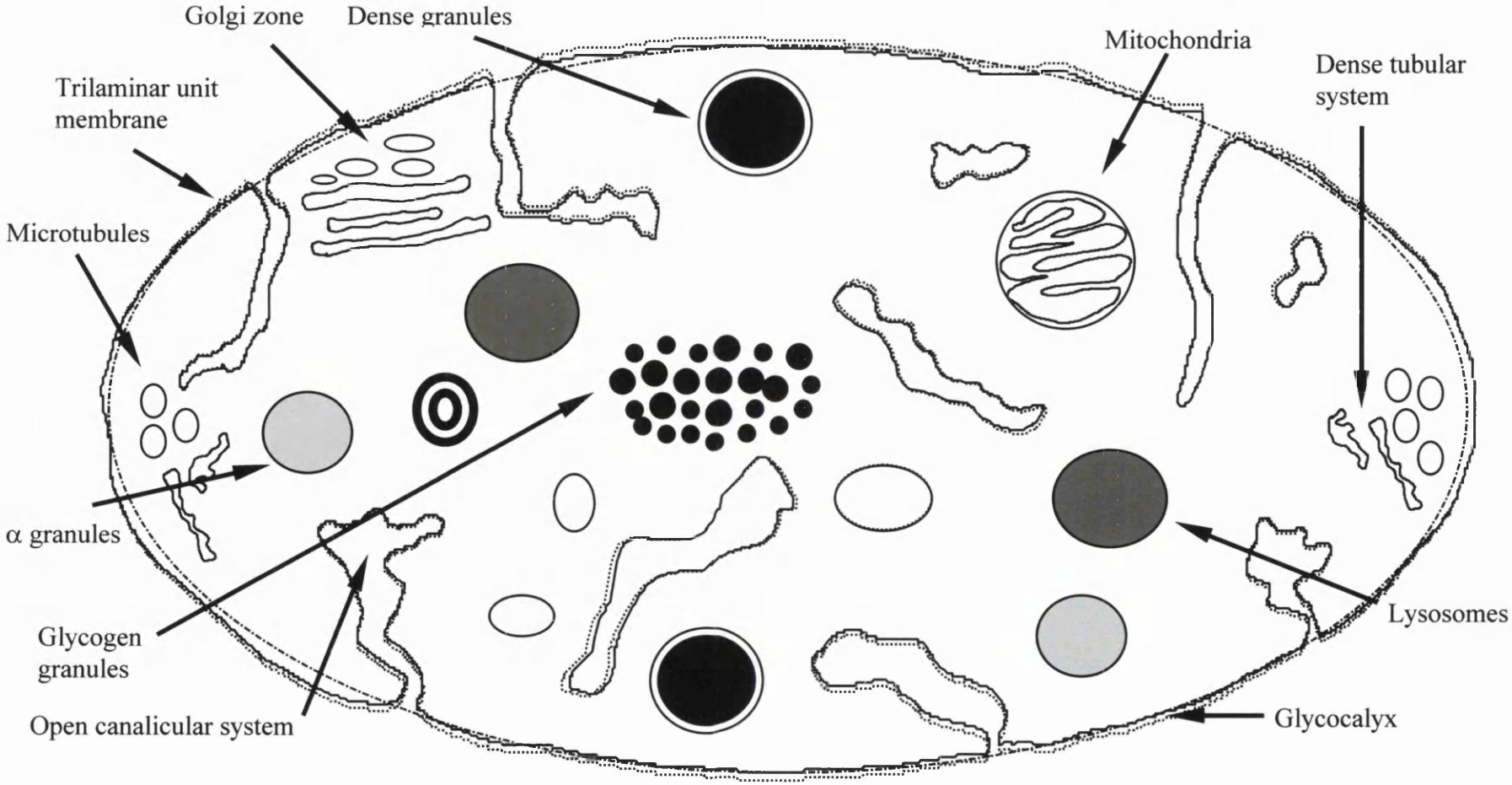

Figure 1.1 *Diagrammatic representation of the ultrastructure of a platelet, as seen in a cross-sectional view*

major features will now be briefly described. On electron microscopy, a fuzzy coat or **glycocalyx** extending 14 to 20 nm from the platelet surface can be seen. It is composed of membrane glycoproteins, glycolipids, mucopolysaccharides, and adsorbed plasma proteins (Ware & Coller 1995). The glycocalyx has a net negative surface charge due to sialic acid residues in the proteins and lipids; the charge is thought to minimise attachment of circulating platelets to each other. The platelet **plasma membrane** is a typical trilaminar membrane composed of cholesterol, glycolipids, and glycoproteins embedded in a phospholipid bilayer (Ware & Coller 1995). It contains the sodium and calcium ATPase pumps that control the platelets ionic environment. About half of the platelets total phospholipid content is contained in the plasma membrane, where it is asymmetrically organised - the negatively charged phospholipids are almost exclusively present in the inner leaflet, whereas the others are more evenly distributed (Ware & Coller 1995). Negatively charged phospholipids (especially phosphatidylserine) are able to accelerate several steps in the coagulation sequence, in particular the conversion of coagulation factor X to Xa and prothrombin to thrombin. The **open canalicular system** (OCS) is an elaborate series of conduits that begin as indentations of the plasma membrane and course throughout the interior of the platelet. It is in direct continuity with the plasma membrane and the external milieu, and serves several functions. It provides a mechanism for entry of external elements into the interior of the platelet, and also provides a potential route for the release of granule contents to the outside; however there is some controversy regarding the relative frequency with which secretion occurs via the open canalicular system versus direct granule fusion with the plasma membrane (Ware & Coller 1995). The OCS also represents an extensive internal store

of membrane which may be used in the processes of filopodia formation and platelet spreading after adhesion. The membrane of the OCS serves as a storage reservoir for plasma membrane glycoproteins that increase on the platelet surface after activation (Stenberg & Hill 1998). The **dense tubular system** is a closed-channel system of narrow, membrane-limited tubules, which is residual smooth endoplasmic reticulum from the megakaryocyte. It is involved in the regulation of intracellular calcium transport, as it has been reported to selectively bind, sequester, and release divalent cations after activation (Stenberg & Hill 1998). It is also the site of prostaglandin synthesis in the platelet. A circumferential band of **microtubules** below the plasma membrane maintain the platelet discoid shape (White 1993) and they are present primarily in their polymerised form in unstimulated platelets. Platelet activation results in microtubule disassembly, then reassembly; such alterations in the marginal microtubule bundle result in platelet shape changes. A network of short **actin filaments** below the plasma membrane make up a **membrane cytoskeleton,** which may cooperate with coils of the circumferential microtubules to maintain platelet discoid shape (White 1993). The cytoskeleton is associated with the cytoplasmic domains of transmembrane glycoproteins, and so may play a role in moving certain receptors from the surface to the interior of platelets and vice versa via the open canalicular system. It may also be important in platelet spreading after adhesion.

Platelets contain four distinct populations of **granules: α-granules, dense bodies, lysosomes and peroxisomes.** **α granules** are the most abundant granules, numbering about 50 to 80 per platelet (Ware & Coller 1995). They contain numerous contents, including platelet-specific proteins (β Thromboglobulin [βTG], platelet factor 4 [PF4]); adhesive glycoproteins (von Willebrand factor [vWF], fibrinogen,

thrombospondin, vitronectin); coagulation factors (Factor V, Protein S); mitogenic factors (platelet-derived growth factor, epidermal growth factor, transforming growth factror-β); fibrinolytic inhibitors; albumin; immunoglobulins; and granule membrane proteins such as P-selectin and GMP-33. Small amounts of virtually all plasma proteins are taken up into the α granules, and thus the plasma level of these proteins will determine the platelet level. On the other hand, platelet-specific proteins such as βTG and PF4 are concentrated in the α granules. **Dense bodies** are electron dense organelles, and contain a high content of calcium, serotonin, adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) (Ware & Coller 1995). Release of dense granule contents from activated platelets constitutes an important positive feedback mechanism for platelet aggregation, since ADP is a potent platelet agonist, and serotonin is a weak agonist. The dense granule membrane contains P-selectin (CD62p) and granulophysin (CD63) (Stenberg & Hill 1998). **Lysosomes** are small vesicles that contain acid hydrolases including β-glucuronidase, cathepsins, collagenase and elastase. Lysosomal membrane glycoprotein (LIMP-CD63) and lysosomal-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2) become expressed on the plasma membrane after activation. When platelets undergo secretion, lysosomal contents are more slowly and incompletely released than are the contents of α granules and dense bodies (Holmsen *et al*, 1982). Stronger inducers of activation are required to obtain any release of lysosomal contents. **Peroxisomes** are very small organelles, which are relatively few in number in platelets and thought to contribute to lipid metabolism (Ware & Coller 1995).

As well as these platelet-specific granules, the interior of the platelet contains a number of other organelles. There are approximately seven **mitochondria** per

platelet (of relatively small size) which are involved in oxidative energy metabolism, and small particles of **glycogen** which play an essential role in metabolism are also found within the platelet.

1.1.2 Platelet membrane glycoproteins

Many of the key biologic processes regulated by platelets are mediated by platelet membrane glycoproteins. These receptors can receive signals from outside the platelet and send signals inside. Similarly, they can receive signals from inside the platelet that affect its external domains. Most of these proteins have now been cloned and sequenced and can be classified within known gene families. The major platelet membrane glycoproteins are listed below in *Table 1.1.*

Gene family	Glycoprotein	Major ligand	Major function
Leucine-rich glycoprotein	Ib/IX	vWF, thrombin	Adhesion
Integrin			
VLA-antigen	Ia/IIa	Collagen	Adhesion
Cytoadhesin	IIb/IIIa	Fibrinogen, vWF, fibronectin, vitronectin	Aggregation
Selectin	GMP-140	Oligosaccharide chain of leukocytes	Cell-to-cell interactions
Immunoglobulin-domain	PECAM-1		Cell-to-cell interactions
	FcγRII	IgG (Fc fragment)	

Table1.1 *Platelet membrane glycoproteins, their ligands and their major functions*

Glycoprotein (GP) Ib/V/IX complexes mediate the adhesion of unstimulated platelets to vWF bound within the subendothelium of damaged vessels. They first stop, then anchor the platelet to the subendothelium under the high-shear conditions of

the microcirculation. Under flow conditions, this attachment leads to platelet activation, granule secretion and platelet aggregation (Moake *et al*, 1988; Chow *et al*, 1992). There are approximately 25,000 GPIb molecules per platelet (Ware & Coller 1995). The **GPIIb/IIIa complex** is the dominant platelet receptor with approximately 40,000 to 80,000 receptors on the surface of a resting platelet. GPIIb/IIIa has a low affinity for binding soluble fibrinogen on resting platelets, but when activated, platelets can bind fibrinogen relatively strongly. Activation induces changes in the GPIIb/IIIa receptor itself that are responsible for this change in fibrinogen-binding affinity (Ware & Coller 1995). It can also bind molecules such as fibronectin, vWF, vitronectin and thrombospondin. The Arg-Gly-Asp (RGD) sequence is the integrin recognition sequence which is known to be present on numerous adhesive proteins including fibrinogen, vWF, thrombospondin, laminin, vitronectin and collagen, and the GP IIb/IIIa complex recognises the RGD sequence within these proteins. **GP Ia/IIa** (also referred to as very late antigen-2 VLA-2), is widely distributed on different cell types and can mediate platelet adhesion to collagen. **Granule membrane protein-140 (GMP-140, also known as CD62p or P-selectin)** is a glycoprotein located in the membrane of platelet α-granules as well as in the Weibel-Palade bodies on endothelial cells. It is important in platelet-leukocyte binding and will be discussed in further detail later. **PECAM-1 (platelet-endothelial cell adhesion molecule-1)** is a 130 kDa glycoprotein of platelets and endothelial cells which belongs to the immunoglobulin superfamily. Its exact function in platelets is uncertain, but it may function as a vascular cell adhesion molecule (Nurden & Nurden 1993). **The class II human immunoglobulin G receptor (FcγRII)** is widely distributed on haemopoietic cells. The FcγRII on platelets may bind immune

complexes generated in certain diseases, and may also be involved in providing a second binding site for antibodies that have bound to platelets via their antibody-binding site (Ware & Coller 1995). FcγRII expression on platelets shows considerable variation among individuals. Two allotypic forms of human FcγRIIa have been distinguished by the presence of either an arginine (Arg 131) or histidine (His 131) at amino acid position 131. The Arg/Arg 131 phenotype binds human IgG_2 and IgG_3 only weakly compared with the His/His 131 phenotype, suggesting that FcγRIIa phenotype may govern FcγR-mediated processes in vivo in humans (Rascu *et al*, 1997).

1.1.3 Platelets and haemostasis

1.1.3.1 Primary haemostasis

When the endothelial cell surface of a blood vessel is injured or becomes disrupted, a platelet and fibrin haemostatic plug is formed. The platelet mediated events (so called 'primary haemostasis') which result in haemostatic plug formation are: **adhesion, shape change, secretion, aggregation** and expression of **procoagulant activity**. Following is a brief description of these key events.

Platelet adhesion

Platelets do not normally adhere to intact endothelial cells, although platelet 'rolling' along the vessel wall may occur. Following blood vessel injury, platelets come into contact with, and adhere to the vessel wall components, in particular collagen and subendothelial microfibrils. Adhesion to collagen involves interaction between the platelet GPIa/IIa complex, and sites on the α-chains of collagen (Hutton *et al*, 1998). Platelet adhesion to subendothelial microfibrils involves the platelet GPIb/V/IX

complex and domains of high molecular weight vWF multimers (which may be attached to the subendothelial microfibrils or in a soluble form in plasma). This interaction between vWF and GPIb/V/IX is dependent on a high shear rate. Once activated, platelets can also bind vWF through the GPIIb/IIIa complex. Platelet adhesion and occupancy of their receptors by many proaggregatory substances, leads to a series of metabolic processes (to be discussed shortly) which result in shape change, release reaction and aggregation of platelets.

Platelet shape change

Immediately following their adhesion, platelets undergo a change in shape, becoming more spherical and extending long, spiky pseudopods. This enhances the interaction between platelets and it is also accompanied by a reorganisation of the platelet's internal structures. The peripheral band of microtubules undergoes central apposition, which forces granules towards the plasma membrane and the OCS, facilitating secretion of their contents.

Platelet secretion/release reaction

In vitro testing has revealed that platelets undergo a specific release reaction following their adhesion and shape change (Hutton *et al*, 1998). The intensity of this platelet secretion ultimately depends on the initiating stimulus. Lower concentrations of thrombin are required for cleavage of the coagulation factor fibrinogen than are required for induction of platelet α-granule secretion, but higher concentrations of thrombin are required for dense granule or lysosomal enzyme secretion than are required for α-granule secretion (Kaplan 1994). Collagen-induced secretion of α-granule proteins occurs with lower concentrations of stimulus than are required for

dense granule secretion, and even higher concentrations of collagen are required for lysosomal enzyme release.

The process by which platelets respond to external stimuli begins with binding of agonists to one or more platelet receptors. Following binding, the platelet synthesises and/or releases several lipid messengers derived from its membrane structures, which in turn mediate changes in platelet function, either directly or indirectly. One major pathway for the formation of these lipid intermediates involves the activation of guanine nucleotide-binding (G) proteins that are interposed between the receptors and the intracellular effectors (Ware & Coller 1995). To date, all of the platelet agonist receptors that have been well characterised on a molecular basis are linked to G proteins. When an agonist binds to its platelet receptor, G proteins become activated, which in turn activate the enzyme phospholipase C (PLC). PLC then hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2), a quantitatively minor component of the surface membrane of platelets, producing diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3) which are both secondary messengers. IP_3 binds to a receptor in the dense tubular system, causing mobilisation of intracellular calcium, whilst DG activates protein kinase C (PKC) activity. Both calcium (Ca^{++}) mobilisation and PKC activation are necessary for full propagation of the many intracellular biochemical processes associated with platelet aggregation and release of platelet granule contents. Ca^{++} mobilisation results in activation of the enzyme phospholipase A_2 (PLA_2), which releases arachidonic acid from membrane phospholipids, ultimately leading to the generation of stimulatory eicosanoids (prostaglandins and thromboxane A_2) which cause further mobilisation of second messengers, platelet aggregation and release of dense granule contents (Ware & Coller 1995).

Platelet aggregation

A wide variety of agonists are able to induce platelet aggregation, however they act by a final common pathway involving the membrane glycoprotein GPIIb/IIIa. This receptor recognises fibrinogen and other ligands through exposure of specific binding sites on platelet activation. Fibrinogen molecules act as bridges between receptors on adjacent platelets, resulting in platelet attachment to each other. Released ADP and thromboxane A_2 (TXA_2) cause additional platelets to aggregate at the site of vascular injury, and further release of these substances results in secondary platelet aggregation and the formation of a platelet mass large enough to plug the area of injury.

Platelet procoagulant activity

In large vessels, reinforcement of the platelet plug by fibrin is necessary in order to prevent its dislodgement by mechanical forces. Platelets contribute to this consolidation in a number of ways, by generating strong procoagulant activities on their surface. Following platelet activation, negatively charged phospholipids are translocated to the outer surface of the plasma membrane ('flip-flop'). A number of coagulation factors, most notably those that are vitamin K dependent (factors II, VII, IX and X) bind avidly to these negatively-charged phospholipids; this process involves a γ-carboxylated glutamic acid rich domain on these coagulation factors, which binds Ca^{++}. Other coagulation factors including fibrinogen and factors V, VIII and XI and high molecular weight kininogen, also bind to platelets, possibly via specific receptors (Hutton *et al*, 1998). The interaction of clotting factors on the surface of activated platelets protects them from naturally occurring inhibitors and localises them to the point of vascular injury. As well as allowing the binding of coagulation factors, phospholipids are also involved in at least two Ca^{++}-dependent

coagulation reactions. One of these, which involves factors IXa, VIII and X, is known as the tenase reaction. The other, known as the prothrombinase reaction results in the formation of thrombin from the interaction of factors Xa, V and II. These reactions are dramatically accelerated when the platelets have been activated by thrombin or other agonists.

Factor XI is tightly bound to platelets, and can be activated there, either by other contact factors which attach to the negatively-charged phospholipids, or by the trace amounts of thrombin generated via the tissue factor pathway (Hutton *et al*, 1998) (see below).

1.1.3.2 The coagulation cascade

As mentioned above, platelets play an important role in the coagulation pathway which involves a complex series of interactions between zymogens, enzymes, and cofactors leading to the generation of thrombin and a fibrin clot. Historically, the coagulation cascade had been divided into 'intrinsic', 'extrinsic' and 'final common' pathways. In this hypothesis, it was proposed that the intrinsic pathway of coagulation involving exposure of the plasma 'contact factors' (factor XII, high-molecular-weight-kininogen [HMWK], and prekallikrein [PK]) to a surface, was the major initiator of coagulation (MacFarlane 1964; Davie & Ratnoff 1964). Factor XI was then activated as a result of the interaction between these factors, and the cascade proceeded as outlined in *Figure 1.2.* Factor XIa in the presence of Ca^{++} then activated factor IX, which in the presence of factor VIII, Ca^{++}, and membrane phospholipid (PL), activates factor X. In the 'final common pathway', activated factor X (together with factor V, phospholipid and calcium) converts prothrombin into thrombin. Thrombin then acts upon fibrinogen to form fibrin (monomer). Fibrin polymerises and then

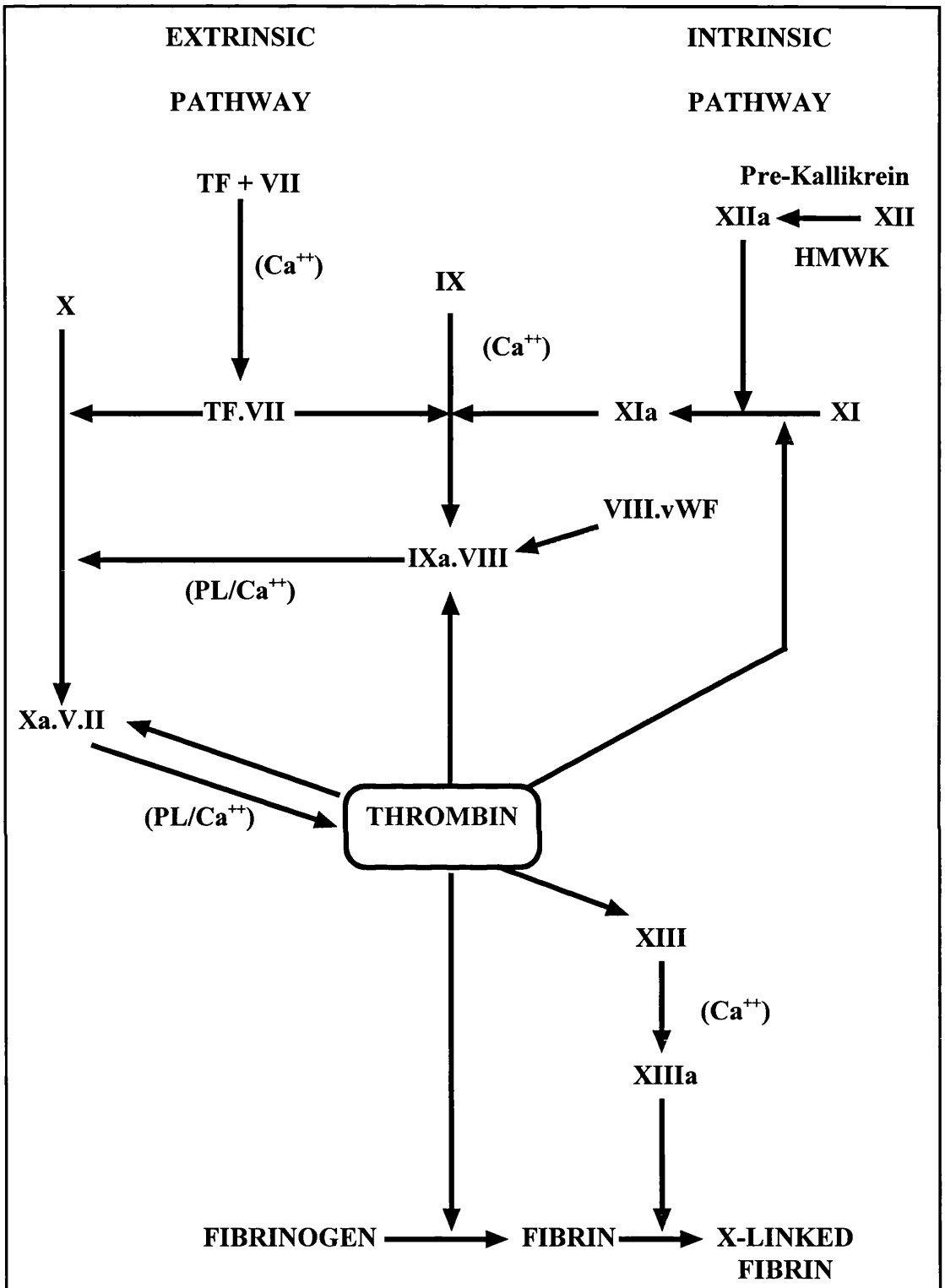

Figure 1.2 *The coagulation cascade demonstrating classical 'intrinsic' and 'extrinsic' pathways*

becomes cross-linked by the action of activated factor XIII. The extrinsic pathway involving factor VII/tissue factor-mediated coagulation, was thought to have an ancillary role since factors VIII and IX (whose deficiencies cause the severe bleeding of haemophilia) were part of the intrinsic pathway. Although this concept has proven invaluable for the *in vitro* testing of haemorrhagic disorders, it is now generally accepted that the initial part of the intrinsic pathway is not important for normal haemostasis at disrupted cell surfaces, and that tissue factor (TF) is responsible for the initiation of physiological coagulation. In this revised hypothesis which is detailed in *Figure 1.3*, coagulation is initiated when damage to a blood vessel exposes blood to the TF produced constitutively by cells beneath the endothelium (Broze Jr 1995). Factor VII or VIIa in plasma then binds to this TF, and the factor VIIa/TF complex activates limited quantities of factor X and factor IX. With the generation of factor Xa, the effect of the plasma proteinase inhibitor tissue factor pathway inhibitor (TFPI) becomes manifest and prevents further production of factors Xa and IXa by factor VII/TF. Thus any further generation of factor Xa must occur via an alternative pathway which involves factor VIIIa and factor IXa. Factor XIa is able to produce any additional factor IXa to supplement that generated by factor VII/TF which is limited due to the presence of TFPI. When this revised model of coagulation was proposed, the mechanism for the activation of factor XI was unclear, however it has since been shown that thrombin is capable of activating factor XI, and that in the presence of a polyanion (e.g. dextran sulphate, heparin), this process is amplified, in part through the autoactivation of additional factor XI by factor XIa (Broze Jr & Gailani 1993). A recombinant, truncated form of human thrombomodulin containing a glycosaminoglycan (GAG) modestly accelerates factor XI activation by thrombin,

and factor XI activation has been more recently shown to occur in experimental human endotoxaemia (Minnema *et al*, 1998).

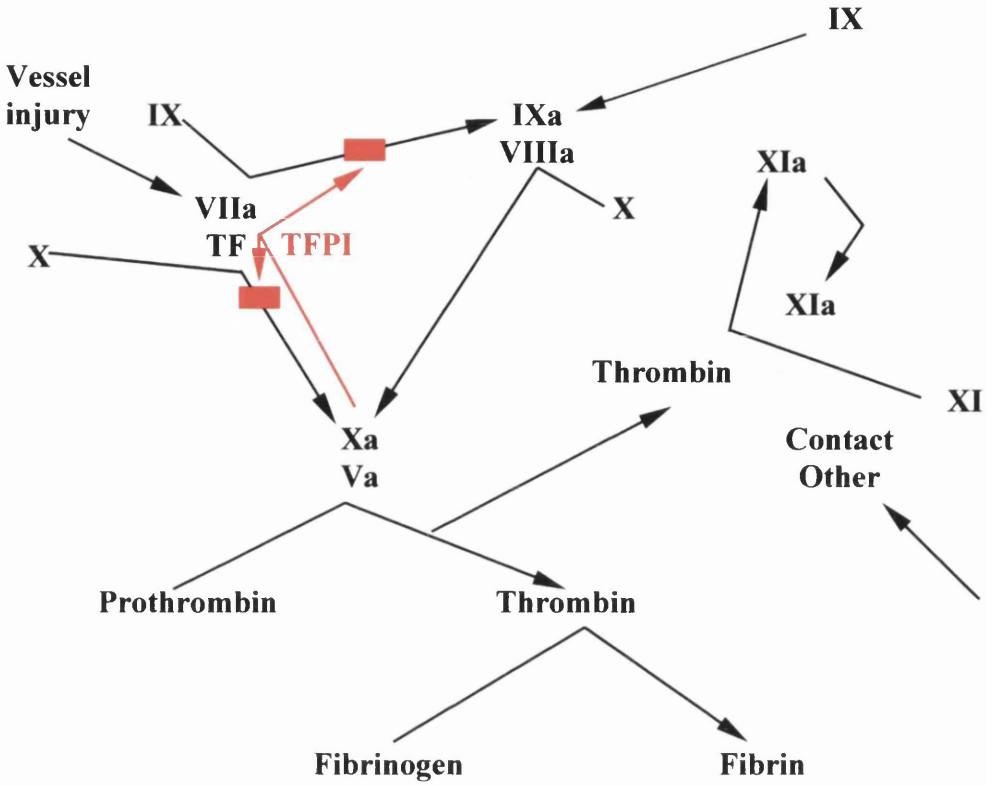

Figure 1.3. *The revised coagulation cascade*
(Note that the requirements for phospholipids and calcium ions for certain of the reactions are not included in this figure)

As can be seen from *Figure 1.2*, thrombin has a central role in haemostasis. It acts upon fibrinogen, and is involved in several other proteolytic processing steps important in regulating blood coagulation. In the procoagulant pathway, Factor V, factor VIII, factor XI and factor XIII are prominent substrates which are cleaved by thrombin. It is also important in the natural inhibitory pathway of coagulation through its effects on protein C (see below). Thrombin activates platelets, and is also involved in cellular growth and migration.

Although the contact factors (HMWK, PK and factor XII) are no longer thought to play a role in the initiation of physiological blood coagulation, it is

becoming increasingly apparent that they may act as antithrombotic and profibrinolytic agents which contribute to the constitutive anticoagulant environment of the intravascular compartment (Schmaier 1997).

1.1.3.3 Inhibitors of blood coagulation

There are several physiological anticoagulants which serve to regulate the coagulation pathways, and some of the major inhibitors will now be briefly described. **Tissue factor pathway inhibitor (TFPI)** has been mentioned previously. It is thought to be synthesised largely by endothelial cells, with a smaller amount being found in platelets. TFPI directly inhibits activated factor X, and in a factor Xa-dependent manner, produces feedback inhibition of the factor VIIa/TF catalytic complex (Broze Jr 1995). **Antithrombin (AT)**, also formerly known as antithrombin III, is a single chain glycoprotein which is synthesised principally in the liver. It forms a stable, 1:1 stoichiometric complex with several serine proteinase coagulation factors - its major substrates are thrombin and factor Xa, but it also forms complexes with factors IXa, XIa and XIIa. Heparin induces a > 2000-fold increase in the rate of thrombin inactivation by AT, such that its action becomes almost instantaneous. Heparin also strongly enhances the speed of neutralisation of factor Xa and to a lesser extent of factor IXa by AT (Hutton *et al*, 1998). **Heparin cofactor II (HCII)** is another protease inhibitor, however its affinity for heparin is signficantly less than that of AT (Rosenberg & Bauer 1994). The specificity of HCII is narrowly restricted to thrombin and it has no inhibitory activity towards other coagulation factors. Polysaccharides such as dermatan sulfate are able to accelerate dramatically the action of HCII (Rosenberg & Bauer 1994), and it appears to be mainly involved in extravascular or tissue associated thrombin inhibition (based on sites of high dermatan sulfate

concentration). The **protein C pathway**, which acts by destroying activated factors V and VIII, is an important regulator of coagulation. **Protein C** is a vitamin K dependent protein which is synthesised in the liver. In order to exert its anticoagulant effect, protein C must first be activated to form **activated protein C (APC).** This is achieved by the action of thrombin, however the reaction is slow in the absence of **thrombomodulin (TM)**, a protein which is an integral membrane constituent of endothelial cells. TM forms a 1:1 stoichiometric complex with thrombin, altering the affinity of thrombin for its various substrates. By complexing with TM, there is a 20,000-fold increase in the rate of activation of protein C by thrombin, and a concomitant loss of procoagulant properties, so that thrombin effectively becomes an anticoagulant. The binding of protein C to TM is enhanced by the recently discovered endothelial protein C receptor (Fukudome & Esmon 1994). APC, aided by its cofactor **protein S** (another vitamin K-dependent glycoprotein) cleaves factors Va and VIIIa into inactive fragments. These reactions take place on a phospholipid surface in the presence of calcium ions. Hereditary deficiencies of the coagulation inhibitors AT, proteins C and S, as well as an inherited resistance to the action of APC due to a genetic abnormality in the factor V molecule (known as factor V Leiden defect), have been described and are associated with a predisposition to venous thromboembolism. Other regulators of coagulation include α_2 macroglobulin, α_1 antitrypsin, α_2 antiplasmin and C_1 esterase inhibitor.

1.1.3.4 Thrombin generation

When prothrombin is activated to thrombin by the prothrombinase complex, the initial cleavage results in an intermediate product, meizothrombin, which is then cleaved to give the products of **thrombin** and **prothrombin fragment 1.2 (F 1+2).** (Factor Xa

acting alone is also able to cleave prothrombin, but this occurs at a much slower rate and with the formation of slightly different intermediate products). When thrombin is formed, it is inhibited by antithrombin (as mentioned previously), forming **thrombin-antithrombin (TAT) complexes** *(Figure 1.4).* Direct measurement of thrombin is unable to detect a subtle degree of thrombin generation, however the quantitation of both prothrombin fragment 1.2 or TAT complexes in plasma allows the monitoring of small degrees of coagulation activation.

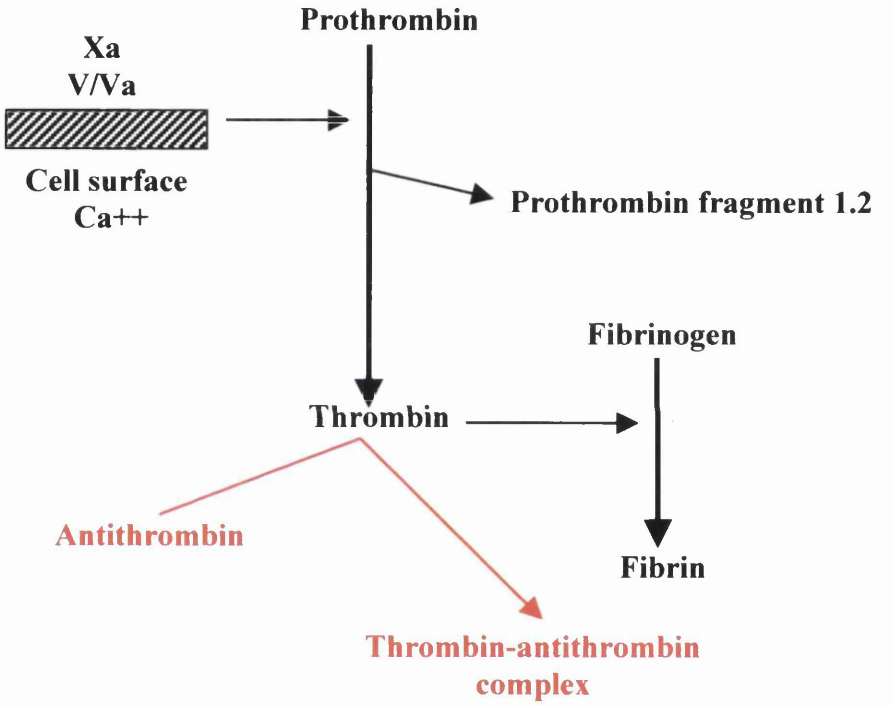

Figure 1.4 *Products of thrombin generation*

1.1.3.5 Fibrin formation and fibrinolysis

Fibrinogen is a glycoprotein which is composed of three pairs of peptide chains (Aα, Bβ, and γ) connected by disulphide bonds to form a symmetrical dimer. Thrombin binds to the fibrinogen central domain liberating fibrinopeptides A and B from the α

and β chains of fibrinogen respectively, to form fibrin monomer which undergoes end-to-end polymerisation to form long fibrin strands which become insoluble. The resulting fibrin clot is stabilised by the factor XIIIa-dependent formation of covalent bonds between α and γ chains of adjacent fibrin strands. Blood cells become trapped within the developing fibrin clot, and the strands complex with platelet aggregates as they form to give a primary haemostatic plug.

Fibrinolysis is initiated by plasminogen activators, enzymes that convert the zymogen plasminogen to the fibrinolytic enzyme plasmin. Plasmin may be activated by tissue plasminogen activator (tPA), urokinase-type plasminogen activator (uPA, urokinase), a contact factor dependent mechanism, or by exogenous (therapeutic) activators such as streptokinase. The fibrin clot is broken down into progressively smaller soluble fragments by the action of plasmin. Plasmin cleaves both fibrin and cross-linked fibrin at a number of sites, liberating fibrin degradation products (FDP's), however if cross-linked fibrin is cleaved, then larger cross-linked products (such as D-dimers) are generated. The fibrinolytic pathway is regulated by specific protease inhibitors, including plasminogen activator inhibitor-1 (PAI-1) and α_2 antiplasmin. All of these reactions are summarised in *Figure 1.5.*

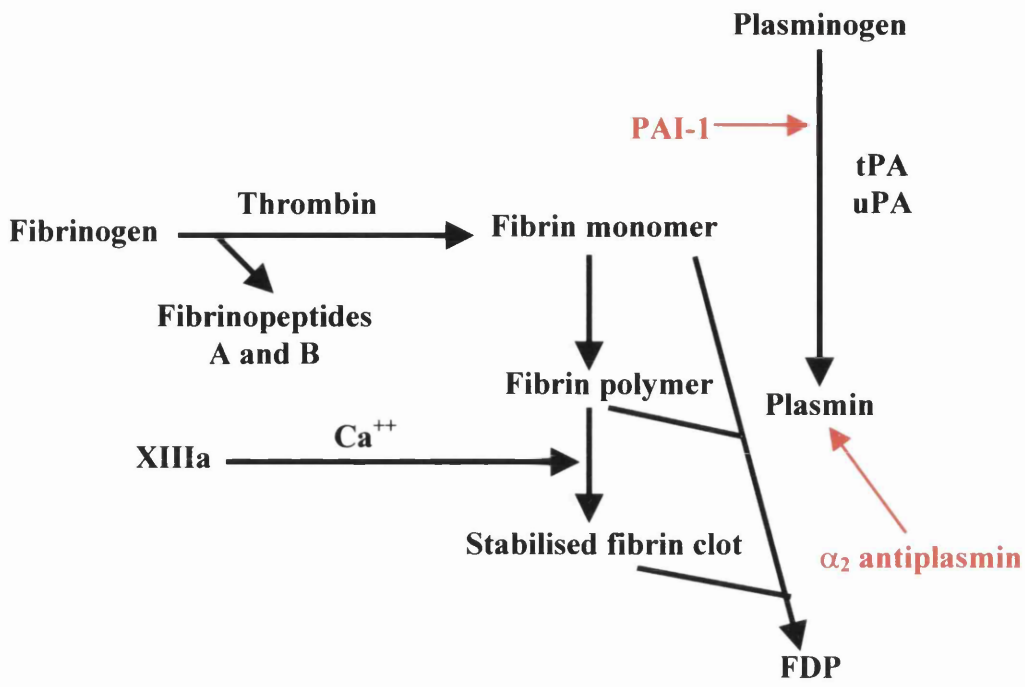

Figure 1.5 *Fibrin formation and the fibrinolytic pathway*

1.1.4 Platelet "activation"

1.1.4.1 General

As previously mentioned, upon activation, platelets lose their discoid shape becoming relatively spherical and extruding long spiky pseudopods. The organelles contract towards the centre, becoming enclosed by a tight-fitting ring of microtubules and microfilaments. Finally the contents of the granules are extruded, and depending on the agonist, aggregation may occur. Platelet aggregometry is a well established method used widely for studying the platelet response to a number of agonists as well as aiding in the diagnosis of platelet dysfunctional disorders. However, platelet aggregation follows many complex biochemical events, and is thus a crude and insensitive marker of platelet activation. Over the years, numerous tests have been

developed to demonstrate 'hyperactivity' of circulating platelets, or to show that platelets have been activated *in vivo* by a prothrombotic process. Following is a brief description of some of the older methods, followed by a more detailed discussion of novel flow cytometric techniques which are currently used to assess platelet activation as they overcome some of the problems associated with the previous methods.

1.1.4.2 Methods for detecting platelet activation

Platelet aggregate ratio - this method was first described by Wu and Hoak in 1974, and then modified by Bowry *et al* in 1985. It quantifies the numbers of small platelet aggregates which may circulate in the blood of patients with thrombotic and hypercoagulable states by comparing the platelet count of blood collected into buffered EDTA either with or without formalin. Rapid handling of the blood after venepuncture is essential, and patients with thrombocytopaenia may give erroneous results.

ADP aggregation threshold - platelets are aggregated to various doses of ADP (as per standard platelet aggregometry), and the ADP dose which just initiates a secondary wave of platelet aggregation is found and known as the threshold dose. A similar threshold may be determined for arachidonate.

Spontaneous aggregation - undiluted platelet rich plasma is stirred in an aggregometer and spontaneous aggregation is measured. The presence of cryoglobulins and cold agglutinins in patients plasma may interfere with the assay.

β-Thromboglobulin (β-TG) - β-TG is stored in the platelet α-granule and is not usually present in significant amounts in normal plasma, thus the presence of raised levels of plasma β-TG may suggest *in vivo* platelet activation. Careful sample collection and handling are essential in order to prevent *in vitro* release of granular

contents, and it is critical to prepare plasma which is devoid of platelets. Blood samples are collected into tubes containing a mixture of anticoagulant and specific inhibitors of platelet secretion (such as theophylline and prostaglandin E_1), and placed immediately on ice. Increased levels of β-TG may occur in renal failure due to decreased catabolism of β-TG by the kidney. False elevations may also occur due to poor sample collection or difficult venepunctures, and comparison of results with platelet factor 4 levels (see below) may be of some help.

Platelet factor 4 (PF4) - PF4 is also stored in platelet α-granules and increased plasma levels suggest *in vivo* platelet activation. The assay method is the same as for β-TG described above. Platelet PF4 is rapidly cleared from the circulation after release, probably by binding to the vascular endothelium. Thus smaller increases in PF4 are seen than for β-TG in thrombotic conditions. PF4 is not affected by renal failure, but increased levels are found after heparin infusion due to displacement of PF4 from the vessel wall. As mentioned, a major problem with both of these assays is in avoiding *in vitro* release during blood collection and plasma separation, and hence the ratio of β-TG to PF4 may help to differentiate between *in vitro* and *in vivo* platelet activation. Kits for measurement of both β-TG and PF4 are commercially available, however, values for these two parameters show significant variability between laboratories (likely due to differences in blood collection and processing).

Thromboxane B_2 (TXB_2) - this metabolite of thromboxane A_2 (which is derived from arachidonate) can be measured by radioimmunoassay and is another way of assessing platelet activation. Both plasma and urinary TXB_2 levels can be measured.

5-hydroxytryptamine release (5-HT) - a radiotracing technique for the measurement of 5-HT release from dense granules can also be used to detect platelet activation.

Many of the assays described above have a number of disadvantages which have limited their utility - plasma separation/washing procedures are often used to prepare platelets, and so they are susceptible to artefactual *in vitro* platelet activation; some assays are insensitive to low levels of platelet activation; radioactive materials are required for some assays which necessitates the requirement for appropriate facilities and precautions; and kits are often very expensive. As a result, flow cytometric methods have developed rapidly in an attempt to overcome many of these shortcomings.

Flow cytometric methods - the introduction of whole blood flow cytometry (Shattil *et al*, 1987) has been a major advance, as it circumvents many of the problems associated with the previously described methods for detecting platelet activation. Minimal manipulation of samples prevents artifactual *in vitro* activation and potential loss of platelet subpopulations. Platelets are analysed in their native milieu of whole blood - including leukocytes and erythrocytes, both of which may affect platelet activation. Only miniscule volumes of whole blood are needed and a spectrum of activation-dependent changes can be detected with a high degree of sensitivity. It is important to note however, that clinical studies using flow cytometric assays performed on washed platelets or platelet rich plasma, are like other assays of platelet function, partially susceptible to artifactual *in vitro* platelet activation as a result of the obligatory separation procedures (Michelson 1996).

Recently, a European Working Group Consensus has been published which outlines procedures for the flow cytometric characterisation of platelet function (Schmitz *et al*, 1998). It recommends the use of whole blood in assays, and makes specific recommendations about preferred anticoagulant, choice of buffer and fixation.

It points out however, that the specific length of time that different activation dependent surface markers remain expressed on the platelet surface *in vivo* has not yet been adequately determined, and so one must interpret any results with this in mind.

One disadvantage of flow cytometry is that it only measures the function of circulating platelets. If activated platelets are rapidly cleared or adherent to the blood vessel walls or other cells, then flow cytometry may underestimate the degree of platelet activation in certain conditions (Michelson 1996).

Following is a detailed discussion of platelet activation markers which can be detected using flow cytometry, and these are summarised *Figure 1.6.*

1.1.4.3 Exposure of granule membrane proteins

Platelet activation by physiological agonists results in degranulation and the incorporation of both α and dense granules and lysosomal membranes into the cell surface membrane. Integral proteins of granule and lysosomal membranes thus become exposed at the cell surface, where they can be measured using specific monoclonal antibodies. The two 'degranulation' markers which are most commonly used to measure platelet activation are **CD62p** and **CD63**.

CD62p which is also known as P-selectin, GMP-140 (granule membrane protein of 140 kDa), or PADGEM protein (platelet activation dependent granule-external membrane protein) is a component of the α granule membrane of resting platelets that is only expressed on the platelet surface membrane after α granule secretion (Stenberg *et al*, 1985; Berman *et al*, 1986). CD62p is also found in the Weibel-Palade bodies of endothelial cells, and is rapidly translocated to the external membrane on cell activation. It is a member of the selectin family of adhesion molecules and recognises carbohydrate ligands related to sialyl Le^x. CD62p

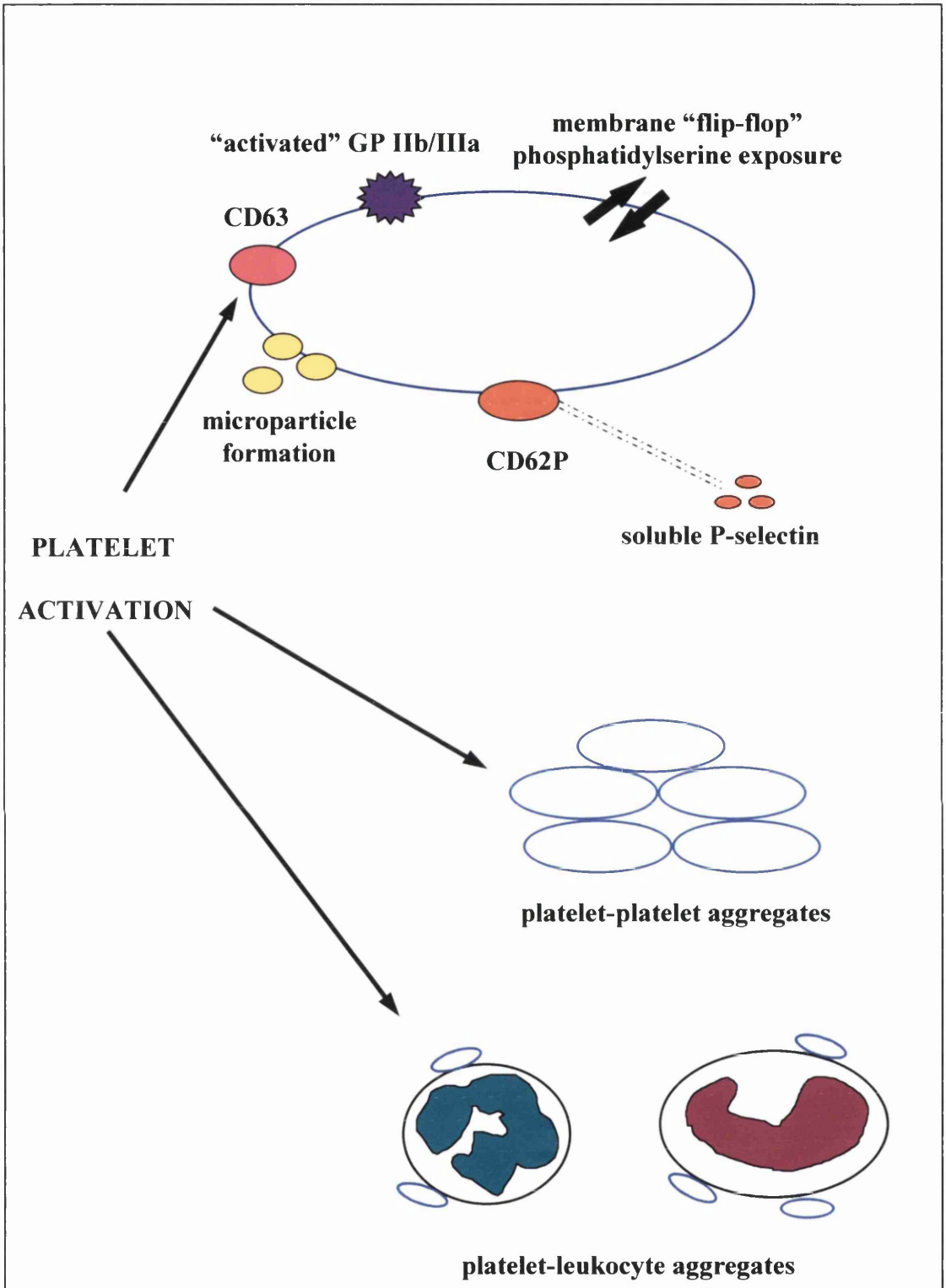

Figure 1.6 *Platelet activation-dependent changes which can be detected using flow cytometric and simple ELISA techniques*

primarily functions as a receptor that mediates the adherence of neutrophils and monocytes (and possibly other leukocytes) to activated platelets and endothelial cells (Larsen *et al,* 1989; Hamburger & McEver 1990). It may be that platelet CD62p serves to localise leukocytes to the site of vascular injury (Larsen *et al,* 1989). It has also been suggested that CD62p mediated binding of activated platelets to monocyte-macrophages in the reticuloendothelial system might represent a means of clearing these cells from the bloodstream, as they are potentially thrombogenic (Celi *et al*, 1991).

A soluble form of P-selectin (sP-selectin) exists and circulates in normal plasma (Dunlop *et al*, 1992). The half-life of sP-selectin is estimated to be 7.2 hours (Wu *et al*, 1993). Although its function is not entirely certain, sP-selectin seems to retain biological activity. *In vitro* studies are conflicting however, as some investigators have found that it enhanced superoxide generation by activated neutrophils at physiological concentrations (Lorant *et al*, 1993), whilst others report that it inhibits superoxide generation by neutrophils stimulated by tumour necrosis factor (Wong *et al*, 1991). Animal studies have demonstrated that circulating degranulated platelets rapidly lose their surface P-selectin to the plasma pool, but continue to circulate and function (Michelson *et al*, 1996). Thus platelet CD62p expression may not always be an ideal marker for detecting circulating degranulated platelets unless the blood sample is drawn within several minutes of the activating stimulus, or there is continuous activation of platelets (Michelson 1996). An increase in the plasma level of soluble P-selectin may be used as a marker of platelet activation, however, it may also possibly reflect the release of P-selectin from activated/damaged endothelial cells (Chong *et al*, 1994).

CD63 or granulophysin is thought to be on the boundary membranes of lysosomes (Nieuwenhuis *et al*, 1987), and dense granules (Nishibori *et al*, 1993) and is expressed on the platelet membrane surface after stimulation. It is a 30-60 kDa integral membrane protein whose function is unknown. It was originally described in platelets but has since been shown to have a wider distribution (Wilkinson 1996). One report suggests that platelet dense granule membranes contain not only CD63 but also P-selectin (Israels *et al*, 1992).

1.1.4.4 Changes in platelet GP IIb/IIIa complex

As well as exposure of these granule membrane proteins on the platelet plasma membrane, changes to some of the membrane glycoproteins may also take place during platelet activation. The GP IIb/IIIa complex undergoes a conformational change when the platelet is activated, and several monoclonal antibodies have been developed which can detect this. One of these is **PAC-1**, an IgM antibody which binds to the GP IIb/IIIa complex on the surface of intact platelets, but only after the platelets have been activated by an agonist (Shattil *et al*, 1985). PAC-1 (which contains the RGD sequence) inhibits platelet aggregation, suggesting that its binding site on the GP IIb/IIIa complex is located at or near the binding site for fibrinogen.

Since platelets are likely to become activated by different agonists in different clinical situations, the pattern of platelet surface activation may vary, and therefore it is probably best to employ a panel of activation-dependent antibodies when studying platelet activation.

1.1.4.5 Platelet-leukocyte aggregates

The recruitment of circulating blood cells to specific sites plays a crucial role in a number of pathophysiological events including inflammation and blood coagulation, and one of the ways in which this may be achieved is by the formation of platelet-leukocyte aggregates. The colocalization of platelets and leukocytes is found at sites of haemorrhage, in vascular grafts, atherosclerotic lesions, and areas of myocardial infarction. Platelet-monocyte interactions may accelerate generation of tissue factor by activated monocytes (Silverstein & Nachman 1987). It is known that thrombin stimulated platelets bind to leukocytes (particularly monocytes compared to neutrophils [Rinder *et al*, 1991a]) and so the analysis of such platelet-leukocyte complexes may provide an additional means of assessing platelet activation.

There are probably several adhesion molecules mediating platelet-leukocyte binding. Of these, P-selectin has been most extensively studied and is known to be involved in the binding of activated platelets and endothelial cells to neutrophils and monocytes (Larsen *et al*, 1989; Geng *et al*, 1990), as well as eosinophils, basophils and a subset of T lymphocytes (de Bruijne-Admiraal *et al*, 1992). The interactions are dependent on divalent cations and can be completely inhibited by a monoclonal antibody against P-selectin. These leukocyte subsets express the P-selectin counterreceptor P-selectin glycoprotein ligand-1 (PSGL-1) (Sako *et al*, 1993), a mucin-like transmembrane protein that contains carbohydrate structures (Moore *et al*, 1994) including sialic acid (Polley *et al*, 1991; Foxall *et al*, 1992) and the Lewis x antigen (Larsen *et al*, 1990) that are required for P-selectin binding. Unlike P-selectin, PSGL-1 is constitutively expressed on the leukocyte surface and there is currently no evidence that its biological activity is altered during leukocyte activity (Furie & Furie

1997). It is possible that there is more than one ligand for P-selectin - some have claimed that sulfatides are also P-selectin ligands (Aruffo *et al*, 1991) and that L-selectin can serve as the P-selectin ligand (Picker *et al*, 1991). The kinetics of platelet adhesion to monocytes and neutrophils is such that monocyte adhesion is favoured over neutrophil adhesion. The inhibition of P-selectin interaction with leukocytes in animal models of thrombosis has revealed the importance of P-selectin binding to PSGL-1 *in vivo*. Anti P-selectin antibodies were able to inhibit leukocyte accumulation in experimental thrombi in baboons as well as attenuate fibrin deposition (Palabrica *et al*, 1992).

The interaction between the cell adhesion molecule CD36 (Glycoprotein IV) and thrombospondin (TSP) may also be important in platelet-monocyte adhesion. CD36 is expressed by platelets, monocytes and microvascular endothelial cells, among other cell types (Daviet & McGregor 1997). It was primarily identified as a receptor for TSP, a major constituent of the extracellular matrix. It has been shown that TSP-dependent platelet-monocyte interactions are mediated by CD36 (Silverstein *et al*, 1989), and this appears to be independent of the CD62p antigen. The platelet glycoprotein IIb/IIIa complex (CD41a) and fibrinogen may also be involved in the adhesion of activated platelets to neutrophils (Spangenberg *et al*, 1993).

'Unactivated' platelets (i.e. those which have not undergone significant α-granule release as determined by CD62p expression) are also capable of binding to isolated neutrophil and monocyte fractions at relatively low numbers of platelet per leukocyte (Rinder *et al*, 1991b). It is possible that fibrinogen-integrin interaction may play a role in this adhesion. The role of leukocyte stimulation in the formation of platelet-leukocyte complexes is not so clear however, with reports of both increased

(Rinder *et al*, 1992; Evangelista *et al*, 1996; Li *et al*, 1997) and decreased (Rinder *et al*, 1994; Lorant *et al*, 1995) complex formation upon leukocyte activation.

Thus, it is likely that multiple mechanisms are responsible for the formation of platelet-leukocyte aggregates and that several receptors are involved. The relative importance of each may vary according to the stimulus responsible for the initial platelet (or possibly leukocyte) activation.

1.1.4.6 Platelet -derived microparticles

'Platelet-dust' was first described by Wolf (Wolf 1967) who found that normal plasma or serum contained coagulant material in minute particulate form, which was sedimentable by high-speed centrifugation, platelet in origin, but distinguishable from intact platelets. These deposits possessed the coagulant properties of platelet factor 3 (phosphatidylserine), and were rich in lipid content. Since then it has been found that such platelet-derived 'microparticles' contain membrane GPIb, IIb and IIIa, and electron microscopy reveals them to be unilamellar microparticles with an average diameter of ~ 0.2μm (Zwaal *et al*, 1992) although they may sometimes be slightly larger.

The significance of microparticles for haemostasis and thrombosis *in vivo* remains somewhat unclear. Microparticles express phosphatidylserine, thereby providing an essential procoagulant surface that supports the formation of activated clotting enzymes i.e. tenase and prothrombinase complexes on membranes (Rosing *et al*, 1985). The importance of this is highlighted by the bleeding disorder known as Scott syndrome, which is associated with an abnormality in platelet microparticle formation and aminophospholipid exposure, with a resulting defect in platelet procoagulant activity (Weiss 1994). However, there is also evidence to suggest that

microparticles may have some anticoagulant properties, since the inactivation of FVa by activated Protein C is increased in the presence of microparticles (Tans *et al*, 1991).

Microparticles may result from a number of mechanisms including the mechanical disruption of platelets due to shear forces (as in cardiopulmonary bypass) or *in vitro* freeze/thaw effects; the activation of platelets during storage or by various agonists (Owens 1994). There is extensive formation of microparticles following exposure to calcium ionophore A23187, collagen plus thrombin, and complement C5b-9; moderate formation after collagen activation; and small numbers are formed after activation by thrombin, ADP or adrenaline alone (Zwaal *et al*, 1992). The process of microparticle formation requires the influx of extracellular calcium. It appears that microparticles generated by different stimuli are distinctive - microparticles induced by C5b-9 do not express fibrinogen receptor, whereas those formed by thrombin do (Sims *et al*, 1989).

Microparticles can be counted in either whole blood or plasma samples using flow cytometric techniques, and so can provide another means for detecting platelet activation.

1.1.4.7 Platelet aminophospholipid exposure

All mammalian cell membranes characteristically display phospholipid asymmetry. In platelets and erythrocytes particularly, the outer leaflet of the lipid bilayer is rich in cholinephospholipids (sphingomyelin and phosphatidylcholine) whereas the inner leaflet is mainly composed of aminophospholipids (phosphatidylserine and phosphatidylethanolamine). It has been suggested that this lipid asymmetry is maintained by the action of two ATP-dependent transporters: aminophospholipid

translocase and a non-specific floppase (Zwaal & Schroit 1997). The aminophospholipid translocase shuttles the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) towards the inner membrane. A non-specific floppase has been found to transport both amino-aminophospholipids and choline-phospholipids from the inner to the outer leaflet. Cellular activation, which is accompanied by an increase in intracellular calcium, induces a fast transbilayer movement of all phospholipids known as 'flip-flop' (Bevers *et al*, 1983). In platelets, the extent of this movement depends on the agonist which is responsible for platelet activation, with agonists such as the calcium ionophore A23187, or collagen plus thrombin having the greatest effects. The most significant change resulting from transmembrane lipid redistribution is PS exposure on the outer leaflet of the cell. This lipid scrambling is thought to be mediated by a lipid 'scramblase'. Surface exposure of PS has two important physiologic functions - firstly, in platelets it plays a pivotal role in promoting blood coagulation primarily through the tenase and prothrombinase reactions; and secondly, it is considered to be a key step in the process of apoptosis as it may represent a surface signal for clearance by macrophages and other cells of the reticuloendothelial system.

PS exposure on cell membranes can be detected by flow cytometry using the glycoprotein annexin V, conjugated to fluorescein. Annexins share the functional property of being able to bind with high affinity to negatively charged phospholipids, but only in the presence of calcium ions.

1.2 Antiphospholipid antibodies and the antiphospholipid syndrome

1.2.1 Background

The first antiphospholipid antibody to be described was the syphilitic antibody 'reagin' in 1906 by Wassermann (Wassermann *et al*, 1906). Following the introduction of the Wassermann test, other procedures were used in the diagnosis of syphilis. One of these was the Venereal Disease Research Laboratory (VDRL) test, in which alcohol extracts of bovine heart were used as the antigen source. Widespread use of syphilis serology led to the identification of individuals who had positive tests, but without any evidence of treponemal infection (Moore and Mohr). These tests were recognised as either acute or chronic biologically false positive tests for syphilis (BFP-STS), and patients with a chronic BFP-STS were often found to have underlying autoimmune disease such as systemic lupus erythematosus (SLE) (Moore & Lutz 1955). Further investigations resulted in the discovery that a phospholipid extracted from bovine heart (given the name 'cardiolipin'), was responsible for the BFP-STS which could occur in patients with SLE (Triplett 1993).

Another member of the antiphospholipid antibody 'family' was described in 1952, when Conley and Hartmann reported the occurrence of a circulating anticoagulant (synonym: inhibitor) in the setting of SLE (Conley & Hartmann 1952). In 1963, Bowie *et al* described thrombosis occurring in association with a circulating anticoagulant in several patients with SLE (Bowie *et al*, 1963). The term 'lupus anticoagulant' was given to this inhibitor by Feinstein and Rapaport in 1972 (Feinstein & Rapaport 1972) to designate non-specific prolongations of whole blood clotting time and PT in patients with lupus. Thus a major paradox of antiphospholipid

antibodies is that they act as anticoagulant *in vitro* whereas they appear to be procoagulant *in vivo*. In 1975, a report of intrauterine death associated with a circulating anticoagulant ('antithromboplastin') was published (Nilsson *et al*, 1975), and following this other groups also reported similar findings (Firkin *et al*, 1980).

In 1983, a complex clinical syndrome characterised by thrombosis, recurrent abortion, neurological disease and antiphospholipid antibodies was first described (Hughes 1983). Since cardiolipin (CL) had been shown to be the antigen that was recognised by the reagin antibody, a solid phase assay for antibodies to CL was developed in 1983 and became an important assay for the detection of APA (Harris *et al*, 1983). In 1987, Harris proposed that the combination of both venous and arterial occlusive events, often accompanied by thrombocytopaenia, in the presence of APA (i.e. anticardiolipin antibody and/or lupus anticoagulant) be termed the antiphospholipid syndrome (APS) (Harris 1987).

In 1989, a multicentre study of 70 patients with so named 'primary' APS was published (Asherson *et al*, 1989). These patients presented typically with a history of deep venous thromboses often accompanied by pulmonary thromboembolism, arterial occlusion (most commonly strokes), or fetal loss. The events were often recurrent and sometimes associated with thrombocytopaenia or haemolytic anaemia. These patients were negative for antibodies to dsDNA and to extractable nuclear antigen (ENA), typical serological features of SLE. Since then, APS has become a well recognised condition throughout the world. The presence of APS in patients with no other evidence of autoimmune disease is known as primary APS (PAPS), whilst APS which is concomitant to other disorders such as SLE, is called secondary APS. APA may also be found in other conditions which are listed in *Table 1.2.*

1.Autoimmune	
	a. Primary
	Do not fulfill criteria for systemic lupus erythematosus
	b. Secondary
	Systemic lupus erythematosus
	Other connective tissue disease
	c. Drug-induced
2. Alloimmune	
	a. Infections
	Viral
	Bacterial
	Protozoal
	Fungal
	b. Malignancies
	Hairy cell leukaemia
	Lymphoproliferative
	Essential thrombocythaemia

Table 1.2 *Classification of antiphospholipid antibodies (from Triplett, 1995).*

Until recently it was presumed that APA were directed against simple anionic phospholipid molecules, however more recent data suggests that the target antigen/antigens are more complex and that some APA require serum cofactors for full expression of their activity *in vitro*. In 1990, three groups reported that the ACL detected by ELISA was not directed against cardiolipin alone, since purified IgG from ACL-positive patients did not bind to cardiolipin unless a plasma protein was present (Galli *et al*, 1990; McNeill *et al*, 1990; Matsuura *et al*, 1990). This protein was identified as β_2-glycoprotein I (β_2GPI) and it is now known that the primary antigen for antibodies in the ACL ELISA system is not cardiolipin, but is usually the plasma protein β_2GPI, bound to cardiolipin (Galli *et al*, 1990; McNeill *et al*, 1990; Matsuura *et al*, 1990). β_2GPI is a 50 kDa glycoprotein which is present in normal plasma at approximately 200 μg/ml, and was first described in 1961 as a perchloric acid soluble

human plasma protein with an unknown function (Schultze *et al*, 1961). It is also known as apolipoprotein H, since it appears in the lipoprotein fractions on ultracentrifugation and activates the enzyme lipoprotein lipase *in vitro*. β_2GPI binds to negatively charged surfaces such as phospholipids, lipoproteins and activated platelets, and has been shown to inhibit prothrombinase activity, ADP-dependent platelet aggregation, as well as the intrinsic pathway of blood coagulation (Schousboe 1985; Nimpf *et al*, 1986; Nimpf *et al*, 1987). However, its biological function remains unknown. ACL of the auto-immune type, which are associated with thrombosis, are generally β_2GPI dependent (Matsuura *et al*, 1990; Keeling *et al*, 1992; Roubey *et al*, 1992; McNally *et al*, 1995a), whereas APA associated with infection bind ACL in a modified CL-ELISA without the need for added β_2GPI (Hunt *et al*, 1992). It has been reported that APA can bind directly to β_2GPI coated on oxidised irradiated wells, but not untreated wells (Matsuura *et al*, 1994). Thus, one theory is that APA bind a cryptic epitope on β_2GPI that is only exposed after interaction with a negatively charged phospholipid or appropriately treated surfaces such as polyoxygenated polystyrene plates treated with gamma irradiation or electrons. An alternative hypothesis is that APA are intrinsically of low affinity and interaction of antibodies with β_2GPI requires capture of this protein to increase its density (Roubey *et al*, 1995). In all, it seems that β_2GPI is the primary antigen with anionic phospholipid membranes acting as a surface to bind antigens *in vivo*.

The nature of the antigens recognised by LA remains unclear, and several protein targets have been proposed, including human prothrombin, β_2GPI, annexin V and possibly other vitamin K dependent proteins such as proteins C and S (Rauch

1998). A number of drugs are known to induce LA's, including chlorpromazine, procainamide, hydralazine, phenytoin and quinidine.

1.2.2 Antiphospholipid syndrome

1.2.2.1 Diagnostic criteria

The antiphospholipid syndrome comprises both clinical and laboratory features which were first proposed as criteria by Harris (Harris 1987) and are listed in *Table 1.3.* In order to be diagnosed as having the antiphospholipid syndrome, patients should have at least one clinical and one serological feature at some time in their disease. An antiphospholipid test should be positive on at least two occasions, more than eight weeks apart (Harris 1987).

Clinical	Serology
Venous thrombosis	IgG anticardiolipin antibody
Arterial thrombosis	IgM anticardiolipin antibody
Recurrent fetal loss*	Positive lupus anticoagulant test
Thrombocytopaenia (immune)	

Table 1.3. *Clinical and serological features of antiphospholipid syndrome*
** usually defined as two consecutive miscarriages or three or more non-consecutive miscarriages*

Recently, it has been suggested that some changes could be made to these criteria (Piette 1998). It has been proposed that clinical features could be categorised as 'major' (i.e. thrombosis or obstetrical criteria) or 'minor' (i.e. livedo, valvular lesions, thrombocytopaenia, chorea and others). Similarly, this approach could be used for serological tests, and it is also important to decide whether or not antibodies

to co-factors (such as β_2GPI) should be included in the diagnostic criteria. For the purposes of this thesis however, I have defined APS according to the criteria listed above.

1.2.2.2 Clinical features

Thrombosis is one of the most common clinical events associated with APA and approximately 30 % patients will have a thromboembolic event. Both venous and arterial thrombi have been reported, with deep venous thrombosis and pulmonary embolism the most frequent. The risk of recurrent thrombosis in patients with APS is high and has been variously reported between 22 to 69 % (Khamashta 1998). Renal vein thrombosis can occur and may sometimes be bilateral. Other sites where thrombosis may occur include renal glomeruli, adrenal glands, and hepatic and mesenteric vessels. The Budd-Chiari syndrome may also occur in APS. Cerebral ischaemia associated with APA is the most common arterial thrombotic manifestation. Other neurological symptoms such as depression, cognitive dysfunction and psychosis have all been associated with APS, and may result from cerebral ischaemia in some, but not all cases. Seizures, chorea and transverse myelitis also appear to be associated with APA but they are more likely to be due to an interaction between APA and central nervous system tissue rather than APA-associated thrombosis (Brey & Escalante 1998). There also appears to be an association between antiphospholipid antibodies and acute myocardial infarction. Several prospective studies have shown that elevated levels of ACL in a non-SLE population imply an increased risk for myocardial infarction, especially in young patients (Vaarala 1998). There is also an association with valvular heart disease, with particularly mitral valve involvement.

Livedo reticularis (cutaneous vascular stasis characterised by a distinctive pattern of cyanosis) is a prominent feature in some patients (Hughes 1984).

Recurrent fetal loss is another major clinical feature of APS. In women with APS who are untreated, a pregnancy loss rate of up to 80-90% (generally during the second or third trimester of gestation) has been reported (Levy *et al*, 1998). Other obstetric manifestations include intrauterine growth retardation, abruptio placenta, prematurity and early severe pregnancy-induced hypertension.

A variable degree of immune-mediated thrombocytopaenia has been reported in as many as 20 - 40% patients with APA (Lechner & Pabinger-Fasching 1985; Galli *et al*, 1996a). Severe thrombocytopaenia (platelet count $< 50 \times 10^9$/l) occurs in about 5 - 10% cases and is seldom associated with haemorrhagic manifestations. Originally, thrombocytopaenia was thought to result from the antiphospholipid antibodies themselves, but now it is considered that coexistent platelet specific antibodies (i.e. those directed against platelet GP's such as IIb/IIIa and Ib/IX) are responsible (Galli *et al*, 1996a; Godeau *et al*, 1997).

A particularly severe, accelerated form of APS known as the 'catastrophic antiphospholipid syndrome' (CAPS), was first described in 1992 (Asherson 1992). Precipitating factors include surgery, drugs, anticoagulation withdrawal and infection. Multiorgan failure is the rule with renal involvement, cardiorespiratory failure, disseminated intravascular coagulation, hypertension, central nervous system involvement and gastrointestinal ischaemia being described. Death occurs in about 50% cases and at necropsy, overwhelming evidence of microthrombotic occlusive disease of small vessels is seen.

1.2.2.3 Laboratory testing for antiphospholipid and related antibodies

Currently, the two antiphospholipid tests which are most widely used, are the lupus anticoagulant (LA) and anticardiolipin (ACL) antibodies. LA prolongs phospholipid-dependent clotting tests, and it is thought that LA may block the *in vitro* assembly and activity of the Xa-Va-Ca++-phospholipid complex (prothrombinase) which is required for the conversion of prothrombin to thrombin (Dahlback *et al*, 1983). The most recently published criteria for the diagnosis of LA (Brandt *et al*, 1995) state that in order to correctly diagnose LA, a sample should demonstrate each of the following features:

- prolongation of at least one phospholipid-dependent clotting test
- evidence of inhibitory activity shown by the effect of patient plasma on pooled normal plasma
- evidence that the inhibitory activity is dependent on phospholipid
- LA's must be distinguished from other coagulopathies that may give similar laboratory results or may occur concurrently with LA's.

In principle, the laboratory tests used to detect LA usually employ a 'screening' stage and a 'confirmation' stage. It is imperative that all plasma samples prepared for LA testing are not comtaminated with platelets and other blood cells, as these will limit the sensitivity of tests (particularly after freezing of samples). The APTT is most frequently used as the initial screening test for LA, and this is performed on freshly prepared patient plasma as well as a mixture (e.g. 50:50) of patient and normal plasma to detect the presence of an inhibitor. The characteristics of the phospholipid component of the APTT reagent appear to be critical in determining its LA sensitivity, and so an additional specific test should be perfromed in suspected cases of LA, even

if the APTT is normal. Once an inhibitor has been identified, it is necessary to determine if it is PL-dependent by performing a confirmatory procedure. This step requires the addition of PL to the patient plasma to detect a relative correction of the abnormal clotting times, and the platelet neutralisation procedure (PNP) is often employed. Tests such as the dilute Russell's Viper venom time (dRVVT) or Kaolin clotting time (KCT) are generally used for the confirmatory stage, and many commercial kits and reagents are currently available for LA testing.

ACL are detected using an ELISA. CL is coated onto mitcrotitre wells and the immobilised CL is incubated with test serum. As mentioned previously, the binding of most ACL antibodies requires the presence of the β_2GPI, which is essential for satisfactory ACL results. The source of β_2GPI is usually fetal calf serum or adult bovine serum in the blocking agent and/or sample diluent. After washing, antibodies bound to the solid-phase CL are detected with an antihuman Ig antibody labelled with an enzyme. This second antibody can be directed against IgG, IgM or IgA and so the isotype of the ACL can be determined. The assay has now been standardised at an International Workshop, and results are expressed as GPL and MPL units. One GPL or MPL unit is defined as the binding activity of 1μg/ml of affinity purified ACL.

In the majority of cases, it appears that LA and ACL antibody activities are probably due to two separate antibody populations. Many individuals with ACL antibodies do not have LA and vice versa (Rosove *et al*, 1989). In patients who do have both activities, there is often a lack of correlation between the levels of ACL and LA (Triplett *et al*, 1988). Both antibodies have markedly different isoelectric points on chromatofocusing separation, and they both appear to be directed against somewhat different combinations of phospholipid moieties and complexes (McNeil *et*

al, 1989). On the other hand, LA activity has been demonstrated in ACL IgG's isolated from the plasma of some patients with concomitant ACL and LA activities (Bevers *et al*, 1991). Thus some investigators have proposed that ACL antibodies can be divided into two distinct subgroups according to their anticoagulant behaviour: ACL type A antibodies, which prolong phospholipid-dependent coagulation reactions by enhancing the anticoagulant effect of β_2GPI, and ACL type B antibodies, that do not promote the anticoagulant activity of β_2GPI (Galli *et al*, 1992).

Antibodies to β_2GPI (in the absence of cardiolipin or other phospholipids) can now be detected using an ELISA system in patients with APS and SLE. A growing body of evidence suggests that there is a relationship between the presence of anti-β_2GPI antibodies and a history of thrombosis (McNally *et al*, 1995a; Martinuzzo *et al*, 1995; Tsutsumi *et al*, 1996; Forastiero *et al*, 1997). However, because there is currently no standardisation with respect to the method, it is difficult to compare studies from different laboratories (Tsutsumi *et al*, 1998).

Anti-prothrombin antibodies are frequently found in patients with APA, and may be responsible for the lupus anticoagulant activity in many cases. A nested case control study has shown that high anti-prothrombin antibody levels implied a risk of deep venous thrombosis and pulmonary embolism (Palosuo *et al*, 1997), whilst another study has reported that a high level of anti-prothrombin antibodies predicted a 2.5-fold increase in the risk of myocardial infarction or cardiac death (Vaarala *et al*, 1996). These studies were retrospective however, and a recently published review suggests that the clinical relevance of anti-prothrombin antibodies remains to be established (Galli & Barbui, 1999).

1.2.2.4 Treatment

The treatment of APS is primarily aimed towards the prevention of thromboembolism and the management of recurrent fetal loss and thrombocytopaenia. Secondary prevention of vascular complications can prove difficult. To date, no prospective clinical trial concerning the optimal management of thrombosis has been published, although the 'Warfarin in AntiPhospholipid Syndrome' (WAPS) study, which is a randomised prospective trial designed to assess the efficacy and safety of high-dose warfarin in controlled conditions, is currently in progress (Finazzi *et al*, 1996). A large retrospective study of 147 patients with APS and a history of thrombosis found that the risk of recurrent thrombosis in APS is high (69% patients had recurrent episodes), and recommended that long term anticoagulation (with or without low-dose aspirin) to maintain the international normalized ratio (INR) at or above 3 is advisable (Khamashta *et al*, 1995). In this series, 29 patients had complications involving bleeding, and in 7 the bleeding was severe. Obviously, the benefits of a 'higher-intensity' regime of warfarin therapy need to be balanced against the risks (which are not insignificant) and it is hoped that the WAPS study will be able to address this issue.

The treatment of choice for APS in pregnancy with previous problems is aspirin and anticoagulation with heparin (both unfractionated and low molecular weight heparins are used). It is important to measure antiXa levels in order to optimise treatment. A randomised controlled trial of low dose aspirin and aspirin plus heparin in 90 pregnant women with recurrent miscarriage associated with antiphospholipid antibodies, found that treatment with aspirin plus heparin resulted in a significantly

higher rate of live births (71%) than that achieved with aspirin alone (41%) (Rai *et al*, 1997).

The thrombocytopaenia of APS requires the same treatment policy as that of immune thrombocytopaenic purpura (ITP). Measures such as steroids, splenectomy, high dose intravenous immunoglobulin, and interferon can be used, and it appears that the presence of APA does not predict the outcome of therapy (Galli *et al*, 1996a).

A number of therapies have been employed to treat catastrophic APS with variable results. These include steroids, cyclophosphamide, plasmapharesis and gammaglobulin (Asherson 1998)

1.2.3 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder with a wide spectrum of clinical features which are listed in *Table 1.4.*

System	Clinical Features
Musculoskeletal	Arthralgia, arthritis, myositis, tendinitis
Cardiorespiratory	Pleurisy, pericarditis, endocarditis, myocarditis, atelectasis,
Nervous system	Polyneuritis, cranial nerve lesions, migraine, spinal cord lesions, cerebritis, epilepsy, chorea, stroke
Urogenital	Cystitis, primary ovarian failure, miscarriages
Renal	Glomerulonephritis, tubular syndromes
Vascular	Raynaud's phenomenon, vasculitis, arterial and venous thrombosis
Constitutional	Fever, fatigue, anorexia, nausea
Haemopoietic	Anaemia (either haemolytic or normochromic-normocytic), thrombocytopaenia, lymphopaenia, leukopaenia, splenomegaly, lymphadenopathy
Mucocutaneous	Mucositis/ulcers, rashes, photosensitivity, alopecia
Ocular	Uveitis, retinal lesions

Table 1.4 *Common clinical features seen in SLE, according to system*

Patients vary with respect to organ involvement and disease severity - some may maintain a relatively normal lifestyle, whilst others will be severely disabled. The disease occurs throughout the world (although there is a predilection for some ethnic groups) and is most commonly found in women of child-bearing age. There is no one single laboratory test or clinical feature which is diagnostic of SLE, hence the American Rheumatism Association (ARA) criteria for disease classification *(Table 1.5)*. Generally, patients with four ARA criteria are classified as having 'definite' lupus, however patients with fewer criteria may still have SLE.

Criterion	Definition
Malar rash	Fixed erythema, flat or raised
Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging
Photosensitivity	By history or observation
Oral ulcers	Observed by a physician, usually painless
Arthritis	Non-erosive; two or more joints
Serositis	Pleuritis or pericarditis
Renal disorder	Proteinuria > 0.5g/24 hour or casts
Neurological disorder	Seizures (not otherwise explained) or psychosis
Haematological disorder	Haemolytic anaemia or leukopaenia or lymphopaenia or thrombocytopaenia
Immunological disorder	Lupus erythematosus cells or anti-native DNA or anti-Sm or biological false positive test for syphilis
Antinuclear antibody (ANA)	ANA positive, in the absence of possible drug induction

Table 1.5 *A summary of the 1982 American Rheumatism Association revised criteria for the classification of SLE*

The disease follows an episodic pattern with 'flares' of disease on a background of chronicity. The most common clinical symptoms encountered in patients include fatigue, skin rashes, arthralgia and arthritis. Nephritis is the manifestation that is most likely to be associated with a significant mortality.

The serologic hallmark of SLE is the presence of autoantibodies against nuclear, cytoplasmic and plasma membrane antigens. Antibodies to double-stranded DNA (dsDNA) are the most prevalent type of antibody in this disorder. Most patients with active disease will demonstrate a significantly raised titre of antinuclear antibodies, associated with low levels of complement C3 and C4.

Therapy is directed against the particular clinical problem, thus a variable number of drug types may be used. These include analgesics, anti-inflammatory agents, antimalarials, corticosteroids and other immunosuppressive agents. Acute flares are usually treated with pulses of methylprednisolone given intravenously.

A review examining the results of 21 studies of 1428 SLE patients has found that 554 possessed APA (39%), and there were 346 patients with histories of thromboses (24%) (McNeil *et al*, 1991). Thromboses were much more frequent in patients with APA (42%) than in those without (13%). Patients who have the secondary antiphospholipid syndrome should also receive treatment with anticoagulant and/or antiplatelet drugs as well as any lupus-specific therapy.

1.3 Antiphospholipid antibodies and thrombosis

1.3.1 General

The strong association between APA and thrombosis suggests a direct pathogenic role of these antibodies in promoting thrombosis. This is quite plausible for several reasons - firstly, the antigens to which they are directed are present in plasma or on cell surfaces that are exposed to plasma and thus circulating antibodies; and secondly, a number of the antigens such as prothrombin are directly involved in haemostasis and thrombosis (Roubey 1998). However, the precise mechanism by which APA may

promote thrombosis is still not resolved, and it is unlikely that any one, single mechanism is responsible for the thrombogenic activity of all APA's.

Thrombosis in APS is probably a 'two-hit' phenomenon (Roubey 1998). This means that autoantibodies (the 'first-hit') are continually present in the circulation, however a local trigger ('second-hit') is required in order to induce thrombosis at a particular site in the vasculature at a particular time. Thus autoantibodies may cause a prothrombotic state in which thrombosis is triggered by local stimuli that would not normally be sufficient to do so. It has been proposed that there are several ways in which autoantibodies may act:

- high affinity, neutralizing autoantibodies may directly inhibit an antigen's function and/or decrease plasma antigen levels via clearance of antigen-antibody complexes
- autoantibodies may cross-link membrane bound antigens causing dysregulation of phospholipid-dependent reactions
- autoantibodies could form immune complexes that are deposited in vessel walls resulting in inflammation and tissue injury
- antibody cross-linking of antigens bound to cell surfaces or cell surface receptors may trigger signal transduction and cellular activation.

Numerous investigators have published studies highlighting abnormalities of many different aspects of haemostasis in patients with APS. Following is a brief summary of some of these reports. A more detailed discussion of the possible role of platelets in APS associated thrombosis follows in section 1.3.4.

1.3.2 APA and haemostasis

APA and tissue factor

Tissue factor (TF) is a small transmembrane protein that is now known to be the major physiological initiator of blood coagulation *in vivo* (Nemerson 1988). Under resting conditions, TF is not normally expressed on intravascular cells, but inflammatory mediators such as interleukin-1 (IL-1), endotoxin, lipopolysaccharides, and tumour necrosis factor (TNF) can induce endothelial cells and monocytes to synthesise and express TF (Colucci *et al*, 1983; Nawroth & Stern 1986). The TF pathway is modulated by the tissue factor pathway inhibitor (TFPI), a proteinase inhibitor.

Numerous investigators have examined the possible role of the TF pathway in the thrombosis of APS. One group have reported that whole IgG from patients with LA activity can induce endothelial cells to express TF (Tannenbaum *et al*, 1986). Patients with APS have been found to have elevated plasma levels of soluble TF and TFPI, and corresponding plasma from these patients induced the expression of both TF antigen and procoagulant activity (PCA) on peripheral blood mononuclear cells (PBMC) in vitro (Amengual *et al*, 1998). These investigators also reported that human monoclonal ACL induced the expression of PCA on PBMC and TF mRNA on PBMC and endothelial cells. In a study which examined monocytes from patients with primary APS and thrombosis, increased expression of TF, TF related procoagulant activity, and cellular and soluble TF antigen were found (Cuadrado *et al*, 1997). However, increased TF production was correlated only with the presence of IgG ACL, and not with IgM or LA. More recently, one group have reported that antibodies to β_2GPI isolated from patients with APS, reduced the PL-dependent inhibitory activity

of TFPI on factor X activation (Salemink *et al*, 1998). Thus these reports suggest that the TF pathway is implicated in the pathogenesis of APA related thrombosis, and that APA may result in overexpression of TF, as well as inhibition of the action of TFPI.

APA and prostacyclin release

Prostacyclin (PGI_2), a major metabolite of arachidonic acid, is secreted by endothelial cells and is a potent vasodilator and inhibitor of platelet aggregation. Membrane phospholipids play a passive role in the synthesis of PGI_2 by supplying arachidonic acid. There are a number of reports which have shown that APA *in vitro* impair PGI_2 release by endothelial cells (McVerry *et al*, 1980; Carreras & Vermylen 1982). Addition of exogenous arachidonic acid abolishes the inhibitory effect in some cases. This inhibition of PGI_2 release by APA is not a consistent finding however (Hasselaar *et al*, 1988). Also, measurements of prostaglandin metabolites *in vivo* in patients with APA are conflicting, with no consistent relationship with thrombosis (Vila *et al*, 1985; Mayumi *et al*, 1991).

APA and fibrinolysis

Fibrinolysis forms an important part of the coagulation cascade, allowing vessels to maintain their patency. It was first suggested that APA may cause abnormalities of fibrinolysis when it was observed that there was an absence of the normal shortening of the euglobulin clot lysis time (ECLT) following venous occlusion in 16 of 28 patients with SLE (Angels-Cano *et al*, 1979). However, in this study, the presence of APA was not documented, and no correlation between thrombosis and abnormal ECLT response to venous occlusion was found. Increased levels of PAI-1 have been reported in patients with SLE (Glas-Greenwalt *et al*, 1984), however it may be that the association is between increased PAI-1 levels and autoimmune disease in general.

Indeed, this has been shown by one group who reported an increase in both tPA and PAI-1 levels in SLE patients, however no difference was found between those SLE patients with APA and those without APA (Keeling *et al*, 1991). Studies examining the effect of APA plasma or purified IgG on spontaneous tPA and PAI-1 release from cultured endothelial cells have failed to demonstrate any change (Cariou *et al*, 1988; Francis & Neely 1989).

Proteins C and S pathway

Both the activation of protein C and the subsequent inactivation of coagulation factors occurs on phospholipid surfaces. Numerous studies investigating proteins C and S in patients with APA have been performed. Although some groups have failed to demonstrate any effect of APA on protein C activation (Keeling *et al*, 1993), others have reported an inhibition of the function of activated protein C in association with APS (Marciniak & Romond 1989; Malia *et al*, 1990; Oosting *et al*, 1993). Plasma and purified IgG fractions from APS patients may reduce the effect of activated protein C in functional assays for activated protein C resistance, mimicking the inherited resistance to activated protein C (Halbmayer *et al*, 1994).

Low levels of free protein S have been reported in patients with APA (Parke *et al*, 1992: Forastiero *et al*, 1994) suggesting that protein S may be involved in the thrombosis of APS. One study has demonstrated that although β_2GPI inhibits the interaction between protein S and C4b-binding protein (C4BP), human monoclonal ACL can reduce this inhibitory activity of β_2GPI regardless of the presence of Ca^{++} (Atsumi *et al*, 1996), suggesting that ACL increases the affinity of C4BP for protein S.

APA and antithrombin III

Antithrombin III (AT-III) is a natural anticoagulant whose action is directed to free circulating serine proteases, and no role for phospholipids in its function has been described. A number of investigators have found that a small proportion of APA positive patients have reduced antigenic or functional levels of AT-III (Boey *et al*, 1984; Hasselaar *et al*, 1989; Lo *et al*, 1990).

APA and factor XII

The contact system protein factor XII is known to be an important component of the fibrinolytic system, and it has been suggested that factor XII deficiency may confer a thrombotic risk. Falsely low levels of factor XII have been documented in patients with LA, and one recently published paper has reported that factor XII antibodies are present in a significant proportion of LA positive patients (Jones *et al*, 1999). The effect of these antibodies on factor XII levels was variable, with some patients having normal factor XII levels, some having a pseudo-factor XII deficiency, whilst others had a true reduction in factor XII levels, presumably through removal of factor XII from the circulation. The clinical significance of these antibodies is currently unknown, however it is possible that they may be involved in the thrombotic risk of APS.

APA and annexin V

Although the physiologic function of annexin V is not yet known, it has potent anticoagulant properties that are based on its high affinity for anionic phospholipids and its capacity to displace coagulation factors from phospholipid surfaces (Andree *et al*, 1992). It has been proposed that autoantibodies may inhibit this physiological

anticoagulant activity of annexin V. One study has found that antiphospholipid antibodies reduce the levels of annexin V and accelerate the coagulation of plasma on the surface of cultured trophoblasts and endothelial cells. This reduction of annexin V on vascular cells may be important in the mechanism of thrombosis and pregnancy loss in APA syndrome (Rand *et al*, 1997). This study also suggests that endogenous annexin V has an antithrombotic role at the interface of trophoblasts and endothelial cells with circulating blood.

APA and β_2GPI

β_2GPI has been shown to inhibit the contact activation of the intrinsic coagulation pathway (Schousboe 1985), platelet prothrombinase activity (Nimpf *et al*, 1986) and ADP-induced platelet aggregation (Nimpf *et al*, 1987). This suggests that β_2GPI may be important in the regulation of anionic phospholipid sites that are required for the efficient activation of factor X and prothrombin (Brighton *et al*, 1996). Hence it has been considered that APA (in particular antibodies to β_2GPI), may induce a prothrombotic state by interfering with the haemostatic regulatory properties of β_2GPI *in vivo*. Indeed, several studies have now been published which indicate that elevated β_2GPI antigen levels and/or the presence of antibodies to β_2GPI are significantly associated with a history of thrombosis and/or recurrent fetal loss in patients APS (McNally *et al*, 1995a; Martinuzzo *et al*, 1995; Forastiero *et al*, 1997). When free β_2GPI antigen is studied in patients with APS, levels are not elevated (McNally *et al*, 1995b). These results suggest that levels of complexed β_2GPI may be increased as a result of immune complex formation, or altered binding to other plasma constituents. Studies of patients with homozygous β_2GPI deficiency have failed to identify β_2GPI

deficiency as an independent risk factor for thrombosis (Banci *et al*, 1992), and so the role of β_2GPI in inducing thrombosis remains unclear.

1.3.3 Mouse models of APS

A number of experimental mouse models for APS have been reported in the literature, and it is hoped that they will result in a better understanding of both the mechanisms and aetiology of this disorder. One group has reported that the intraperitoneal injection of APA from women with foetal loss into pregnant mice resulted in decidual necrosis and foetal loss (Branch *et al*, 1990). Others have shown that the transfusion of peripheral blood lymphocytes derived from a patients with APS and renal dysfunction into SCID mice, induced similar renal pathology in the mouse as in the patient (Levy *et al*, 1996).

The possible thrombotic role for APA has been demonstrated in a mouse model for thrombosis developed by Pierangeli *et al* (Pierangeli *et al*, 1994a). In this model, the effect of APA on clot formation has been studied by recording the kinetics of thrombus formation and the size of the thrombus. In an initial study, mice were immunised (passively) with human IgG from APS patients (IgG-APS), IgG from normal pooled sera (IgG-normals), or saline solution, before having a standardised pinch injury applied in order to induce thrombosis. Results showed that the average clot size was significantly larger in mice immunised with IgG-APS compared to those treated with saline (Pierangeli *et al*, 1994a). The thrombus also persisted longer in a significantly higher number of mice immunised with IgG-APS compared to IgG-normals or saline. This data suggested that IgG-APS may play a role in thrombus formation in humans. A subsequent study found that not only IgG, but also IgM and IgA immunoglobulins from patients with APS could induce the same changes

(Pierangeli *et al*, 1994b). This was also seen when mice were immunised with affinity-purified IgG or IgM anticardiolipin antibodies. More recent work by the same group has demonstrated that ACL antibodies induced by the active immunisation of mice with human β_2GPI or human IgG APA are thrombogenic (Pierangeli *et al*, 1996), and it now appears that it is murine ACL, but not antiβ_2GPI antibodies which are responsible for the thrombogenic properties (Gharavi *et al*, 1998). It is important to remember however, that these findings do not necessarily extrapolate to humans, but nevertheless, they do support the notion that APA are pathogenic.

1.3.4 Platelets and APA

1.3.4.1 APA binding to platelets

Numerous studies have investigated the possibility that APA may bind to platelets, although the findings have not always been consistent. One group found no evidence of APA in the membrane of gel-filtered platelets of patients with APS and thrombosis, (however patients were receiving oral anticoagulants at the time, and there was no evidence of an acute thrombosis) (Biasiolo & Pengo 1993). Others have demonstrated that affinity purified ACL can bind to immobilised platelets bound to a solid phase (Hasselaar *et al*, 1990), even though resting platelets would appear to have little opportunity to bind APA, since their anionic phospholipids are normally located on the inner leaflet of the platelet membrane. Indeed, it has been shown that platelet activation and/or damage is a prerequisite for the binding of APA (Khamashta *et al*, 1988; Mikhail *et al*, 1988; Shi *et al*, 1993). One group reported that ACL will only bind to freeze-thawed but not intact resting or activated platelets (Haga *et al*, 1992).

Studies investigating the binding of β_2GPI and APA to platelets using flow cytometric methods may help to clarify the situation, and one report has found that the exposure of anionic phospholipids upon platelet activation allowed the binding of β_2GPI and in turn, IgG APA to the platelet (Vazquez-Mellado *et al*, 1994). In a physiological situation, platelets are not activated, and β_2GPI is present in plasma without binding to them. However, upon platelet activation and subsequent exposure of anionic phospholipids, β_2GPI can bind.

Discrepancies concerning results of such APA-platelet binding studies may result for a number of reasons - *in vitro* platelet handling and centrifugation may explain the binding of ACL to fixed platelets in the ELISA system (Hasselaar *et al*, 1990), and it is likely that the variable availability of certain 'cofactors' (e.g. β_2GPI, prothrombin) in different experimental conditions influence the reactivity of APA with platelets (Galli *et al*, 1996a).

1.3.4.2 APA and platelet activation

General theory

One model which has been suggested for the pathogenesis of thrombosis in APS draws parallelisms with the thrombosis of heparin-induced thrombocytopaenia (HIT) (Arnout 1996) and is illustrated in *Figure 1.7.* It proposes that as a consequence of an initial damage, activation results in local exposure of negatively charged phospholipids on the surface of platelets, endothelial cells or on trophoblasts. These potentially reactive phospholipids are covered by phospholipid binding proteins such as β_2GPI or prothrombin. If APA are present against such surface bound proteins, they will concentrate on the cell surface, bind to cellular FcγRII receptors, and induce

strong thrombosis promoting modifications. It is known that the interaction of immunoglobulins with platelets can occur by at least three different mechanisms (Chong *et al*, 1995). Firstly, the Fab terminus of the immunoglobulin molecule may bind specific platelet antigens in an antigen-dependent reaction. Secondly, IgG in the form of immune complexes may also bind to platelets via FcγRII. And lastly, immunoglobulin can bind in a nonspecific manner.

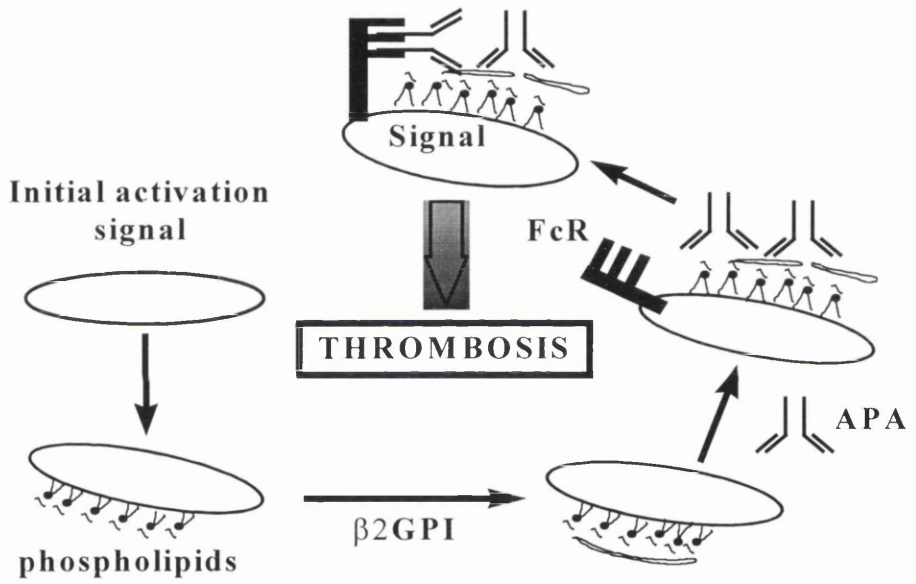

Figure 1.7 *A model for antibody-mediated thrombosis in APS. Mild platelet activation results in phospholipid exposure with binding of phospholipid binding proteins such as β_2GPI. Antiphospholipid antibodies stabilise this interaction via complex formation and via additional FcγRII interactions*

Studies of APA and platelet activation

Many studies investigating both *in vivo* and *in vitro* platelet activation in APS have been published, and only a few of them will be discussed here. Studies published several years ago assessed platelet activation by measuring platelet release products such as βTG, or metabolites of TXA_2, whilst the more recent reports have measured

platelet activation using flow cytometric methods, which are considered to have a number of advantages over the older methods (see section 1.1.4.6).

Studies of *in vivo* platelet activation in APS patients have described increased urinary excretion of TXB_2 metabolites (Forastiero *et al*,1998; Martinuzzo *et al*,1993) an imbalance of thromboxane/prostacyclin biosynthesis in some patients with LA (Lellouche *et al*, 1991), and accelerated spontaneous platelet aggregation (Wiener *et al*, 1991), which support the theory of platelet activation in the development of APS associated thrombosis. On the other hand, others have reported a lack of spontaneous platelet aggregation and in general, normal aggregatory responses in PRP from patients with APA (Diez-Ewald *et al*, 1995). Flow cytometric studies of platelet activation have also reported variable findings. One group have reported significantly higher platelet CD62p values in PAPS patients compared to normals (Fanelli *et al*, 1997), and amongst those with thrombocytopaenia, a significantly increased percentage of CD62p and higher levels of ACL IgG were found compared to PAPS subjects with a normal platelet count. Others have found no significant increase in platelet CD62p in patients with APS (Shechter *et al*, 1998), although there was a significantly lower concentration of serotonin in platelets of APS compared to controls, suggesting that platelets in APS patients circulate in an activated state. Such differences in findings may result from varying experimental conditions e.g. the use of PRP compared to whole blood for flow cytometric studies, and these will be discussed in further detail in the relevant results chapters. A significant increase in the percentage of platelet microparticles has been reported in APA patients compared with normals (Galli *et al*, 1995), however, there was no correlation between

microparticle percentages, platelet number, ACL titre, or degree of prolongation of phospholipid dependent coagulation tests by LA.

Many studies investigating the *in vitro* effects of APA positive plasma/serum or affinity purified IgG on normal platelets or endothelial surfaces have been published. In some cases, there appears to be no effect - both LA positive and LA negative plasmas equally enhanced aggregation of normal platelets when stimulated with low concentrations of ADP (Ichikawa *et al*, 1990). In some experiments, APA binding to circulating platelets was not associated with measurable aggregation abnormalities nor with an increase in platelet CD63 expression (Out *et al*, 1991). A recently published paper also reports that despite the presence of platelet membrane binding IgG in serum from some APS patients, there was no evidence of any associated enhanced platelet-activating ability as measured by microparticle formation and CD62p expression (Ford *et al*, 1998). On the other hand, a large number of reports demonstrate a stimulatory effect of APA on platelets which have been previously 'primed' by a low dose agonist. ACL/β_2GPI complexes from patients with APS when incubated with thrombin activated platelets, resulted in a significantly increased *in vitro* TXB_2 production (Robbins *et al*, 1998). However, there was only a moderate increase in platelet TXB_2 production when ACL alone was used, and none when β_2GPI alone was tested, suggesting that ACL have the ability to bind to PS that is expressed on a procoagulant surface, such as activated platelet membranes. When the effects of affinity purified IgG ACL antibodies from patients with APS and with syphilis on platelet CD62p expression and aggregation were compared, it was found that in the presence of low concentration agonists, all 6 IgG-APS induced platelet aggregation and activation, but none of the IgG-syph had this effect (Campbell *et al*,

1995). In the absence of agonists, only half of the IgG-APS caused platelet aggregation and none caused platelet activation. Platelet CD62p expression was significantly augmented by ACL+LA+ plasma/purified IgG from APA positive patients when used in combination with low concentrations of ADP (Nojima *et al*, 1999). However, this did not occur with plasma samples that were ACL-LA+, ACL+ LA- or ACL-LA-. The effects of murine monoclonal antibodies to β_2GPI (IgG_1 subtype) on platelet function have also been studied (Arvieux *et al*, 1993). These antibodies induced platelet aggregation and secretion responses provided that subthreshold concentrations of weak agonists were added. However, the $F(ab')_2$ fragments of one of the antibodies failed to activate platelets and inhibited the responses to the whole antibody, which suggests that this process depends on MoAbs binding to platelets through both Fab and Fc domains. This was subsequently confirmed by the suppression of platelet responses upon pretreatment with the anti-FcγRII Mab IV.3. Plasma from PAPS patients has also been reported to promote platelet thrombi formation on denuded subendothelium under flow conditions (Reverter *et al*, 1995).

Thus although the findings appear to be inconsistent, there is certainly a large body of experimental literature which supports the theory that platelet activation occurs in APS and may result from a platelet-activating ability of APA, but only in the presence of a sufficient agonist. This is in agreement with the previously mentioned 'two-hit' hypothesis of thrombosis in APS which suggests that autoantibodies (the 'first-hit') are continually present in the circulation, however a local trigger ('second-hit') is required in order to induce thrombosis at a particular site, and at a particular time (see section 1.3).

1.4 Aims of thesis

The primary objective of this thesis was to investigate *in vivo* platelet activation in patients with APS, in order to examine their possible role in thrombosis. Two major groups of patients were selected for study: patients with well documented PAPS, and patients with SLE (many of whom also had secondary APS). Thrombin generation and endothelial cell activation were also measured in an attempt to explore a possible relationship between all parameters and to assess the possible contribution of thrombin to any observed platelet activation. Screening for some of the inherited risk factors of thrombophilia (factor V Leiden and prothrombin gene mutation) was also performed.

Since many of the patients were receiving specific antiplatelet, anticoagulant and/or immunosuppressive therapy (which could not be discontinued for the study), the potential effects of such treatment on all parameters assayed could be investigated. Similarly, any correlations between particular clinical features (e.g. history of thrombosis) and the results of laboratory testing were also examined.

Since the pathogenesis of thrombosis in APS still remains unclear, an attempt was also made to investigate the possibility that APA may directly activate platelets, by performing a series of *in vitro* experiments using purified IgG from selected APS patients.

2. General Methods

All chemicals were obtained from Merck (BDH) Ltd. or Sigma-Aldrich Chemical Co. Ltd. unless otherwise stated. A list of addresses of companies used to supply reagents, equipment and consumables used in this thesis is given in appendix 1. In this chapter they will be cited by name only.

2.1 Sample collection and separation

2.1.1 Sample collection

Blood was collected from normal, adult, apparently healthy, subjects, as well as from patients routinely attending Haemostasis and Rheumatology Outpatients Clinics at University College London Hospitals, London. All patients gave verbal consent for any extra blood samples collected and sample collection was performed by myself. All samples used for platelet activation studies were collected from subjects who were rested for approximately 20 minutes prior to venepuncture in order to minimise platelet activation, and an atraumatic technique was used. Blood was collected from the antecubital fossa using a 21G Butterfly® needle and Vacutainer® system with a luer adaptor (Becton Dickinson). The first 4.5 ml blood collected was drawn directly into a sterile Vacutainer® containing 0.054 ml EDTA (K_3) to determine a full blood count; the next 4.5 ml was collected into a sterile Vacutainer® containing 0.5 ml 0.105 M sodium citrate for platelet activation markers and reticulated platelet analysis. Further tubes of citrated and non-anticoagulated blood were then collected for all other tests.

2.1.2 Sample separation

All plasma, serum and buffy coat samples were separated within 2 hours of collection unless otherwise stated.

Platelet poor plasma (PPP) was prepared from citrated anticoagulated blood. Samples were initially centrifuged at 2000 *g* for 15 minutes at room temperature. The top two-thirds plasma was carefully removed with a plastic pastuer pipette and centrifuged again for 15 minutes at 2000 *g*. PPP was removed, aliquoted into polypropylene tubes and frozen at -70°C. Plasma for routine clotting assays and most ELISA's was only spun once.

Platelet rich plasma (PRP) was prepared from citrated anticoagulated blood. Samples were centrifuged at 180 *g* for 10 minutes at room temperature. PRP was removed and stored at room temperature in a capped tube. PPP was then added to the sample to adjust the platelet count to 250×10^9/l.

Serum was collected from blood which had been allowed to clot at 37°C for 30 minutes before being centrifuged at 2000 *g* for 15 minutes at room temperature. Serum was removed with a plastic pasteur pipette, aliquoted into polypropylene tubes and frozen at -70°C.

Buffy coat was collected from citrated/EDTA anticoagulated blood which had previously been centrifuged to obtain PPP. Buffy coat was carefully collected, aliquoted into polypropylene tubes and frozen at -70°C.

2.2 Flow cytometry

2.2.1 General principle

Flow cytometry involves particles or cells flowing through a laser beam. The signals which are subsequently produced provide information about the particle. A particle suspension is inserted into the flow chamber through which sheath fluid also flows. The pressure of the sheath fluid against the suspension aligns the particles in single file - known as hydrodynamic focusing. Inside the flow chamber, particles are passed through a laser beam and light is scattered in different directions. Light from the laser beam deflected by the surface of the particle is called **forward scatter (FS)** and reflects particle size. Laser light deflected off internal structures or granules within a particle supplies the majority of **side scatter (SS)** light. Dyes may also be bound within or on the surface of a particle. These dyes absorb the laser light energy and emit **fluorescence** of different colour wavelengths which can then be detected. The different colours are separated by filters and directed to sensors called photomultiplier tubes (PMT's). The PMT converts the light to a voltage pulse which rises and falls with the amount of light entering it.

Table 2.1 lists some of the most common fluorochromes used in flow cytometry. In the majority of cases they are conjugated to monoclonal antibodies for the detection of cell surface antigens.

Fluorochrome	Emission wavelength	Fluorescence	Fluorescence detector
Fluorescein isothiocyanate (FITC)	525 nm	Green	FL1
Phycoerythrin (PE)	575 nm	Orange	FL2
R-phycoerythrin-Cy5 (RPE-Cy5)	670 nm	Red	FL4
Thiazole orange (TO)	533 nm	Green	FL1

Table 2.1 *Fluorochromes used in flow cytometry*

Antibody binding can be expressed as either mean particle fluorescence intensity (MFI) or the % of platelets staining positive for a particular antibody (based on an analysis marker placed to the right of the negative control fluorescence histogram). Advantages and disadvantages exist for both ways. Expressing results as the % of platelets is simpler, and unlike MFI, it is independent of variation in signal amplification (e.g. as a result of changes in PMT voltage or gain), since the isotypic control signal increases in proportion with the test sample (Michelson 1996). However, it is important to realise that antibody positive platelets may have very little antigen expressed on their surface.

2.2.2 Quality control

In order to ensure inter-assay sample reproducibility, Flow-Check™ Fluorospheres (Beckman Coulter) were run daily. These 10 μm fluorescent beads which emit within the range from 525 nm to 700 nm when excited at 488 nm, are used to verify instrument optical alignment and fluidics. A half peak coefficient of variation ≤ 1.6% on the relevant histograms was accepted.

2.2.3 Platelet surface markers CD42b, CD62p and CD63

Principle

Platelets can be easily identified from other circulating blood cells by the use of monoclonal antibodies to platelet-specific antigens such as glycoprotein Ib (CD42b). Platelet activation results in degranulation and the incorporation of both granule and lysosomal membrane proteins into the cell surface membrane, where they become exposed and can be used as markers of platelet activation. These include both CD62p and CD63 which have been discussed previously in the introduction.

Reagents

- Monoclonal antibodies anti-CD42b-PE, anti-CD62p-PE, and either anti-CD63-PE or anti-CD63-FITC (Immunotech, Beckman Coulter)
- Isotype controls IgG1-PE and IgG1-FITC (Immunotech, Beckman Coulter)
- HEPES buffered saline (HBS) - NaCL 0.145 mol/l, KCl 5 mmol/l, $MgSO_4$ 1 mmol/l, HEPES 10 mmol/l, pH 7.4
- Formaldehyde solution - made up to 0.2% in 0.145 mol/l NaCL, and filtered with a Sartorius Minisart® 0.2 μm syringe filter (Fisher Scientific) prior to use. A fresh aliquot was made each day.
- Reagents for validation - thrombin related activation peptide (TRAP) (Sigma-Aldrich)

Method

Whole blood (5 μl) was incubated with HBS and either 5 μl or 10 μl of test antibody/corresponding isotype control to make a final total volume of 50 μl. In the initial study (Chapter 3), anti-CD63-PE was used, whereas in subsequent work, anti-CD63-FITC was used. After 20 minutes incubation at room temperature, the samples

were fixed in 0.5 ml of 0.2% formalin saline prior to flow cytometric analysis. All samples were analysed on the flow cytometer within three hours of venepuncture.

Flow cytometry was performed on a Coulter® Epics® XL-MCL flow cytometer (Beckman Coulter). Platelets were identified by the characteristic "platelet cloud" seen on the scatterplot of log forward scatter versus log side scatter. An analysis region was drawn around the platelet cloud, and a listmode gate was employed to exclude cells other than platelets from being analysed. The monoclonal antibody anti-CD42b was used to confirm the identity of platelets within the cloud, and the analysis region was adjusted for each sample to ensure that > 95% of the particles analysed were positive for anti-CD42b. Antibody binding was expressed as the percentage of platelets staining positive for a particular antibody. An analysis marker placed to the right of the negative control fluorescence histogram was set so that 0.5% events were positive. A total of 10,000 events per sample were analysed.

The following figures illustrate examples of typical scatterplots (*Figure 2.1*) and histograms (*Figures 2.2, 2.3, and 2.4*) obtained during an analysis for platelet activation markers.

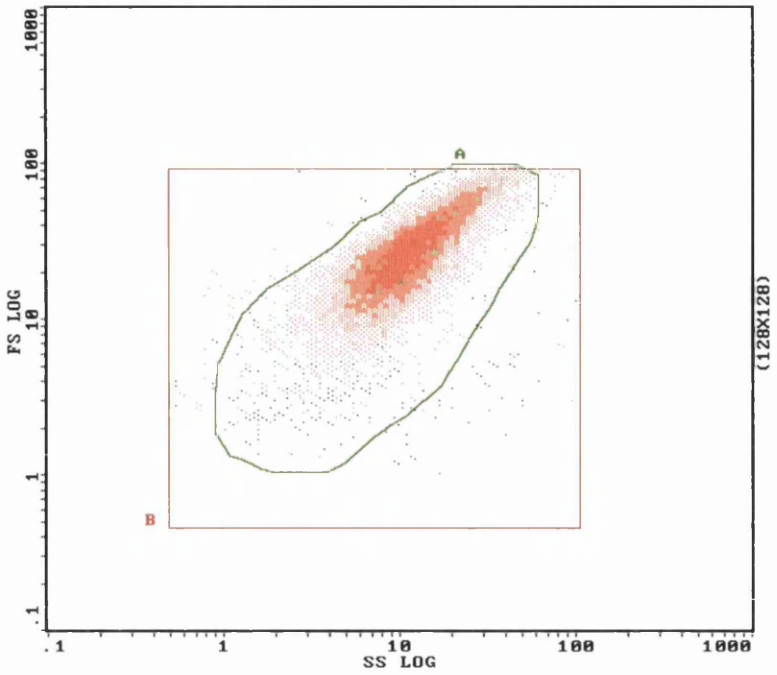

Figure 2.1 *Scatterplot demonstrating typical platelet cloud seen on log side scatter (SS LOG) versus log forward scatter (FS LOG) histogram. Region 'A' is an analysis gate drawn around the platelet cloud. Only these events are further analysed for fluorescence. Region 'B' is a listmode gate which excludes other cells (erythrocytes and leukocytes) from being analysed*

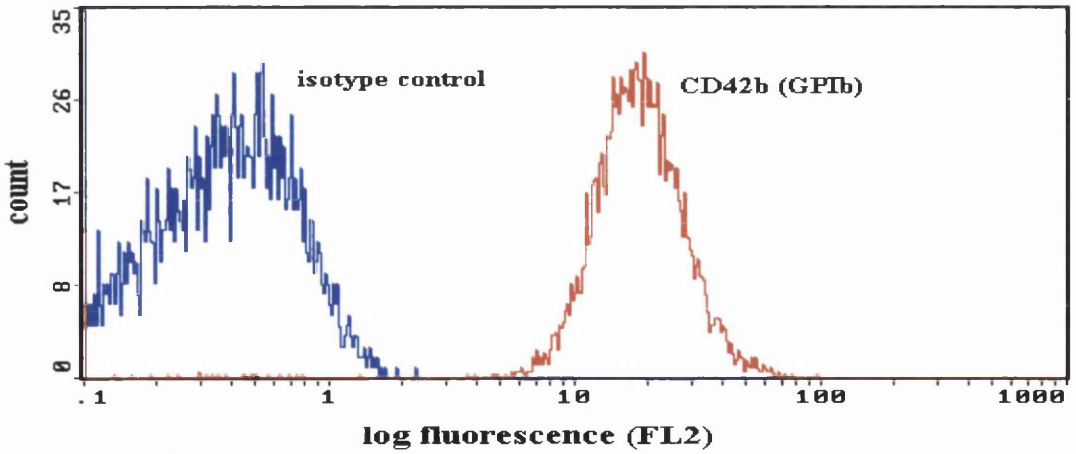

Figure 2.2 *Histogram demonstrating typical fluorescence profiles for platelets stained with isotype control or monoclonal CD42b-PE antibody. Approximately 99% events occurring in Region 'A'(Figure 2.1 above) are positive for platelet specific CD42b in this example. Count = cell number*

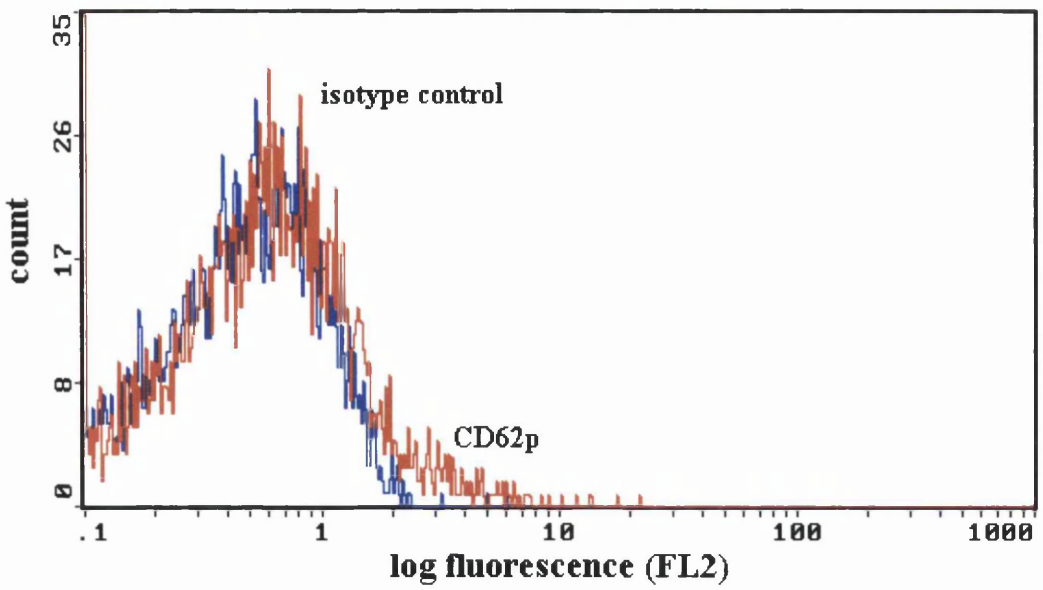

Figure 2.3 *Histogram demonstrating typical fluorescence profiles for platelets stained with isotype control or monoclonal CD62p-PE antibody. Approximately 2-3% platelet specific events are positive forCD62p in this example. Count = cell number*

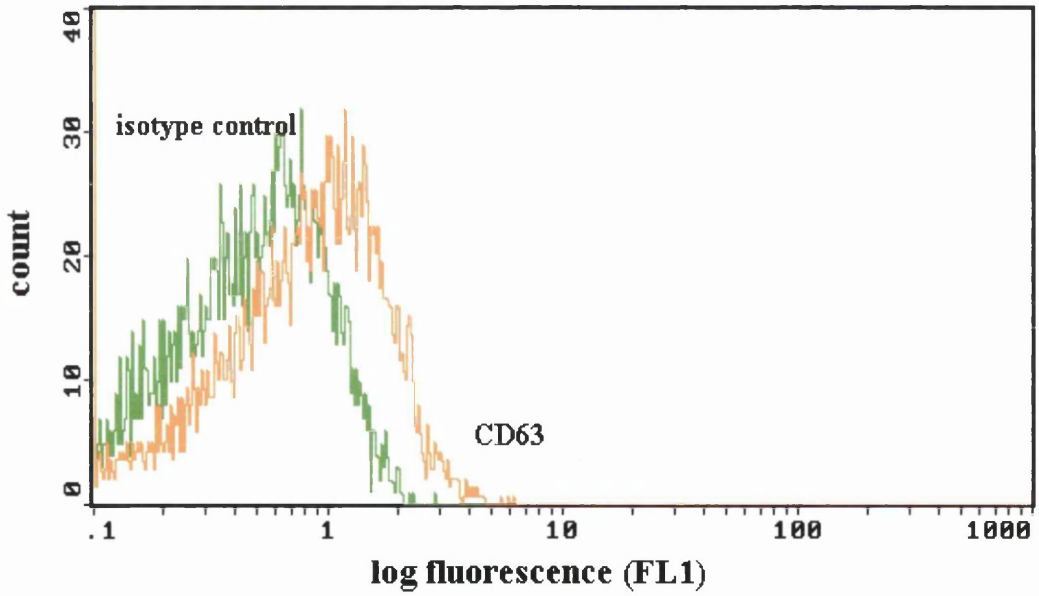

Figure 2.4 *Histogram demonstrating typical fluorescence profiles for platelets stained with isotype control or monoclonal CD63-FITC antibody. Approximately 5 % platelet specific events are positive for CD63 in this example. Count = cell number*

The initial work in my thesis (Chapter 3) was performed on a different flow cytometer to the remainder of my thesis work (Chapters 4 and 5), although they were both Coulter® Epics® XL-MCL flow cytometers (Beckman Coulter). Separate normal ranges were obtained for each machine since there were differences in the 'sensitivity' of both FL1 and FL2 detection between the two cytometers. Also, as mentioned previously, anti-CD63-PE was used for work conducted in Chapter 3, whereas anti-CD63-FITC was used for the remainder of the thesis work. In my initial study (Chapter 3), 12 healthy individuals were used as controls. The reference ranges (based on geometric mean ± 2 SD of log normalised data) are 0.8% - 13.9% for CD62p, and 6.2% - 16.3% for CD63. In Chapter 4, 20 healthy individuals were used as controls, and the reference ranges (based on geometric mean ± 2 SD of log normalised data) are 0.3% - 3.6% for CD62p, and 1.3% - 8.3% for CD63. Only within assay (intra assay) CV's were performed for platelet activation markers, as blood samples could not be stored and analysed at another time. The intra assay CV for measuring both CD62p and CD63 expression in resting samples was 7.1%.

Method Validation

The ability of these antibodies to detect fully 'activated' platelets was verified in control experiments using 80 μM thrombin related activation peptide (TRAP) as an agonist. TRAP is a peptide fragment of the 'tethered ligand' receptor for thrombin. It directly activates platelets without resulting in a fibrin clot. One advantage of TRAP is that it can be used in the absence of the peptide Gly-Pro-Arg-Pro (GPRP) which is required to inhibit clot formation and platelet aggregation when thrombin is the agonist, however, it may not reflect all aspects of thrombin induced platelet activation, because the tethered ligand receptor may not be the only platelet receptor for thrombin

(Yamamoto *et al*, 1991). Each tube contained only one antibody - there was no double labelling of samples. This is because the spectral overlap of emission between fluorescence pairs such as FITC and R-PE is only incompletely corrected by hardware compensation (Schmitz *et al*, 1998). Significant artefacts can occur in the quantitative analysis of weakly expressed antigens, especially when antigens which are expressed more abundantly are stained simultaneously. This is the case in a typical assay where only weakly expressed antigens such as CD62p or CD63 are analysed simultaneously with CD42b. The extent of the artefactual error in quantitation of a weakly expressed antigen will depend on the degree of the spectral overlap between the flurochromes used.

2.2.4 PAC-1 binding

Principle

PAC-1 is a monoclonal antibody that recognises an epitope on the glycoprotein (Gp) IIb/IIIa complex of activated platelets at or near the fibrinogen binding site. The Gp IIb/IIIa complex is located on the surface membrane of resting platelets, and activation induces a calcium-dependent conformational change in Gp IIb/IIIa that exposes a ligand binding site.

Reagents

- Monoclonal antibody PAC-1-FITC (Becton Dickinson)
- Isotype control IgM-FITC (Sigma-Aldrich)
- Dulbecco's phosphate buffered saline (PBS) without calcium, magnesium and sodium bicarbonate - KCl 2.7 mmol/l, KH_2PO_4 1.5 mmol/l, NaCL 0.137 mol/l, Na_2HPO_4 8 mmol/l (Life Technologies)

- Formaldehyde solution - made up to 0.2% in 0.145 mol/l NaCL, and filtered with a Sartorius Minisart® 0.2 μm syringe filter (Fisher Scientific) prior to use. A fresh aliquot was made each day.

Final method

Within 5 minutes of venepuncture, whole blood (5 μl) was aliquoted into a tube containing 5 μl of test antibody/isotype control and 40 μl Dulbecco's PBS. After 20 minutes incubation at room temperature, the samples were fixed in 0.5 ml of 0.2% formalin saline. Flow cytometry was performed as described previously for platelet surface markers. The normal reference range for PAC-1 expression (based on the geometric mean ± 2 SD of log normalised data) was 2.1% - 6.4%. The intra assay CV for measuring PAC-1 expression in resting samples was 8.2%.

Method development

The method described previously for measuring CD42b, CD62p and CD63 expression had been used in our laboratory for some time, with very reproducible results. However, I encountered a number of problems with this method for measuring PAC-1 binding. In essence, this method resulted in extremely high levels of PAC-1 binding (approximately 70 to 90%) in resting samples. As a result, a number of different variables were systematically examined in order to develop a valid and reproducible assay. These experiments are briefly summarised below.

1) Effect of buffer type -

As stated above, when HEPES buffered saline was used as the sample buffer, very high levels (approximately 70 to 90%) of PAC-1 binding were found in resting samples. A number of other buffers were tried - Isoton® II (Beckman Coulter), an azide-free balanced electrolyte solution, resulted in very low levels of basal PAC-1

binding (approximately 1 to 2%), but there was no significant increase when platelets were maximally stimulated with TRAP; both phosphate buffered saline (PBS) and Modified Tyrode's buffer resulted in elevated levels of basal PAC-1 binding (approximately 40 to 60%), however when these buffers were used, TRAP stimulation resulted in maximal PAC-1 binding.

2) Effect of tube type -

The samples were routinely incubated in polystyrene tubes. I considered that this might be affecting the level of PAC-1 binding, however there were no differences in PAC-1 binding results when polypropylene tubes were substituted instead.

3) Effect of time delay after venepuncture -

Although antibody staining was performed as quickly as possible after the blood sample had been collected, a delay of up to 60 minutes could sometimes occur - particularly when samples were collected "off site". This did not appear to be a significant problem with the established methods for CD42b, CD62p and CD63 measurement, however it was considered as a variable which may be important in PAC-1 binding. To address this issue, blood collected into citrate anticoagulant was aliquoted into tubes containing PBS (together with PAC-1 antibody) at timepoints 5, 15, 30 and 60 minutes after venepuncture. (These formal timepoint experiments were not performed on samples using HEPES buffered saline, as previous work had shown levels of approximately 85% PAC-1 binding when blood was immediately diluted into this buffer). All samples were also activated with TRAP to assess whether this method would detect fully activated platelets. The results are shown in *Figure 2.5* and demonstrate that there is a time dependent increase in PAC-1 binding which becomes apparent at approximately 60 minutes following venepuncture. PAC-1

binding appears to be stable for at least 30 minutes following venepuncture, thus enabling the use of this antibody as another marker of platelet activation.

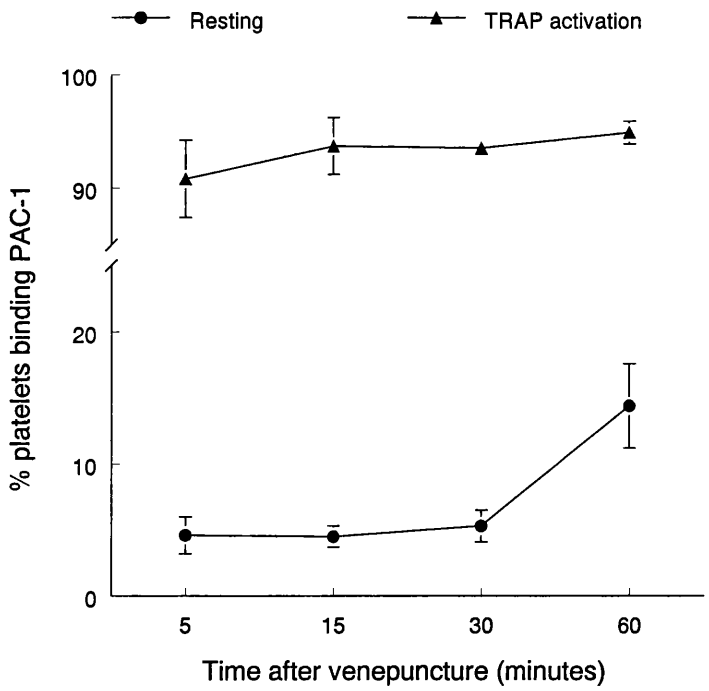

Figure 2.5 *Effect of time delay between venepuncture and sample antibody staining on % platelets (both resting and TRAP activated) binding PAC-1 in controls (n=3).* Mean ± SD are indicated.

2.2.5 Annexin V binding

Principle

Annexin V is a member of the "Annexin" family of proteins, which share the functional property of binding with high affinity to negatively charged phospholipids such as phosphatidylserine (PS), but only in the presence of Ca^{++} ions. PS is normally restricted to the inner leaflet of the plasma membrane of blood cells, such as platelets. Upon cell activation, PS is exposed to the outer cell surface, where it may be detected by phospholipid binding proteins such as Annexin V. Fluorescein-conjugated Annexin V can be used for the flow cytometric detection of PS exposure on platelets.

Reagents

- Annexin V Fluos (FITC) (Boehringer Mannheim) - made up in 10 µl aliquots and stored at -20°C
- HEPES buffered saline (HBS) - NaCL 0.145 mol/l, KCl 5 mmol/l, $MgSO_4$ 1 mmol/l, HEPES 10 mmol/l, pH 7.4
- K_2EDTA-HBS - 5 mmol/l K_2EDTA made up in HEPES buffered saline
- Ca-HBS - 2.5 mmol/l $CaCL_2$ made up in HEPES buffered saline
- Reagents for validation - Calcium ionophore A23187 (Sigma-Aldrich) which was solubilised in DMSO, made up in 10 µl aliquots and stored at -20°C; Gly-Pro-Arg-Pro (GPRP) (Sigma-Aldrich), made up in 10 µl aliquots and stored at -20°C.

Method

The method used was adapted from several which had been recently published (Ruf *et al*, 1997; Metcalfe *et al*, 1997). Whole blood (5µl) was diluted initially in 43 µl either K_2EDTA-HBS (used as the control) or Ca-HBS before adding Annexin V Fluos (2µl) to both tubes. After a 20 minute incubation at room temperature, the samples were then diluted in 0.5 ml of either K_2EDTA-HBS or Ca-HBS before being analysed on the flow cytometer according to the protocol outlined in section 2.2.3. The normal reference range for annexin V binding was based on the geometric mean ± 2 SD of log normalised data from 20 normal individuals, and was 1.3% - 6.0%. The inter assay CV for measuring annexin V binding to resting platelets was 6.5%.

Method validation

The ability of Annexin V Fluos to detect platelets with maximal phospholipid exposure was verified in control experiments using the calcium ionophore A23187. In these experiments, maximal platelet phospholipid exposure was induced by first

incubating whole blood with the calcium ionophore A23187 (final concentration 3 μM) in the presence of calcium (final concentration 2.5 mM) and the peptide Gly-Pro-Arg-Pro (GPRP) (final concentration 2.5 mM) which is used to inhibit thrombin-induced clot formation and platelet aggregation. After a 10 minute incubation at 37°C, aliquots of this sample were then analysed for annexin V binding as detailed above. This method resulted in levels of approximately 75 to 80% annexin V 'positive' platelets. Formalin saline was not used as a fixative in this assay as it resulted in a dramatic increase in annexin V binding with levels of 90 to 95% positivity in resting samples.

2.2.6 Platelet-leukocyte aggregates

Principle

Platelet-leukocyte aggregates are thought to be another marker of platelet activation. Adhesion of platelets to white cells has been studied previously by quantitating the heterotypic conjugates (rosettes) that form after mixing suspensions of isolated platelets and polymorphonuclear leukocytes, usually using microscopy (Jungi *et al*, 1986). Recently, several authors have published methods for the detection of these conjugates using flow cytometric methods (Redlich *et al*, 1997; Li *et al*, 1997). It has been reported that *in vitro* sample manipulations such as sample fixation, erythrocyte lysis, and in particular, repeated centrifugation, can cause artefactual platelet-leukocyte aggregates (Li *et al*, 1997).

Reagents

- Monoclonal antibody anti-CD45-RPE-Cy5 (Dako)
- Monoclonal antibody anti-CD42b-PE (Immunotech, Beckman Coulter)

- Isotype control IgG1-PE (Immunotech, Beckman Coulter)
- HEPES buffered saline (HBS) - NaCL 0.145 mol/l, KCl 5 mmol/l, $MgSO_4$ 1 mmol/l, HEPES 10 mmol/l, pH 7.4
- Hanks Balanced Saline Solution 10% (HBSS) without calcium, magnesium and phenol red (Life Technologies)
- Formaldehyde solution - made up to 10% in distilled water
- Distilled water
- Fixative - made up of 0.5 ml of 10% formaldehdye solution, 0.6 ml of HBSS 10%, and 0.9 ml of distilled water.

Final method

Within 5 minutes of venepuncture, whole blood (25 µl) was aliquoted into a tube containing 65 µl HBS and 5 µl of anti-CD45-RPE-Cy5 plus 5 µl of either anti-CD42b isotype control or test antibody. After 10 minutes incubation at room temperature, the samples were fixed in 84 µl of fixative, and following a further 10 minute incubation, 840 µl of distilled water was added in order to induce erythrocyte lysis. After a further 10 minutes, samples were ready for analysis on the flow cytometer, and were stable for up to 3 hours (at least).

A protocol was designed in which only CD45 positive events (i.e. leukocytes) were further analysed. On the initial scatterplot, log fluorescence (FL4 - RPE-Cy5) was plotted against side scatter, and a listmode gate was employed to exclude any red cell debri from being analysed *(Figure 2.6A).* Events in the gated region were then analysed further, and on a scatterplot of side scatter versus forward scatter, three distinct populations of leukocytes were identified *(Figure 2.6B).* Gates were drawn around each of the three regions labelled 'C' (granulocytes), 'D' (monocytes) and 'E'

(lymphocytes) and any platelet-specific events (i.e. CD42b positive) occurring within these separate regions were then measured. A minimum of 1,000 monocyte events per sample were analysed. An example of the histograms obtained from a typical PLA analysis is shown in *Figure 2.7.* Platelet events (in association with a leukocyte) are detected by FL2, and as can be seen from the histograms, <5% of all circulating leukocytes (from normal, resting subjects) are complexed with platelets. The normal reference range for platelet-leukocyte aggregates was based on the geometric mean ± 2 SD of log normalised data from 20 normal individuals, and was 2.5% - 4.8% for platelet-granulocyte complexes; 3.4% - 9.1% for platelet-monocyte complexes; 1.9% - 4.6% for platelet-lymphocyte complexes; and 3.0 - 5.5% for total platelet-leukocyte aggregates. The inter assay CV's for measuring platelet-leukocyte aggregates in resting samples were 2.1% - 10.5%.

In setting up this protocol for analysing PLA, a number of issues were addressed. Firstly, due to the presence of dual antibodies in the same sample tube (i.e. anti-CD45-RPE-CY5 and anti-CD42b-PE), a degree of compensation was required as there is some overlap between the fluorescence profiles of the antibodies used. This did not effect the sensitivity of the analysis. Also, the possibility that some of the observed PLA may be due to dual events (i.e. the passage of a single leukocyte and a single, unattached platelet through the flow chamber simultaneously) had to be considered. An experiment in which washed leukocytes stained with anti-CD45 and washed platelets stained with anti-CD42b were then mixed together and analysed on the flow cytometer, found a minimal number of dual events occurring, particularly if samples were analysed on a 'low' flow rate.

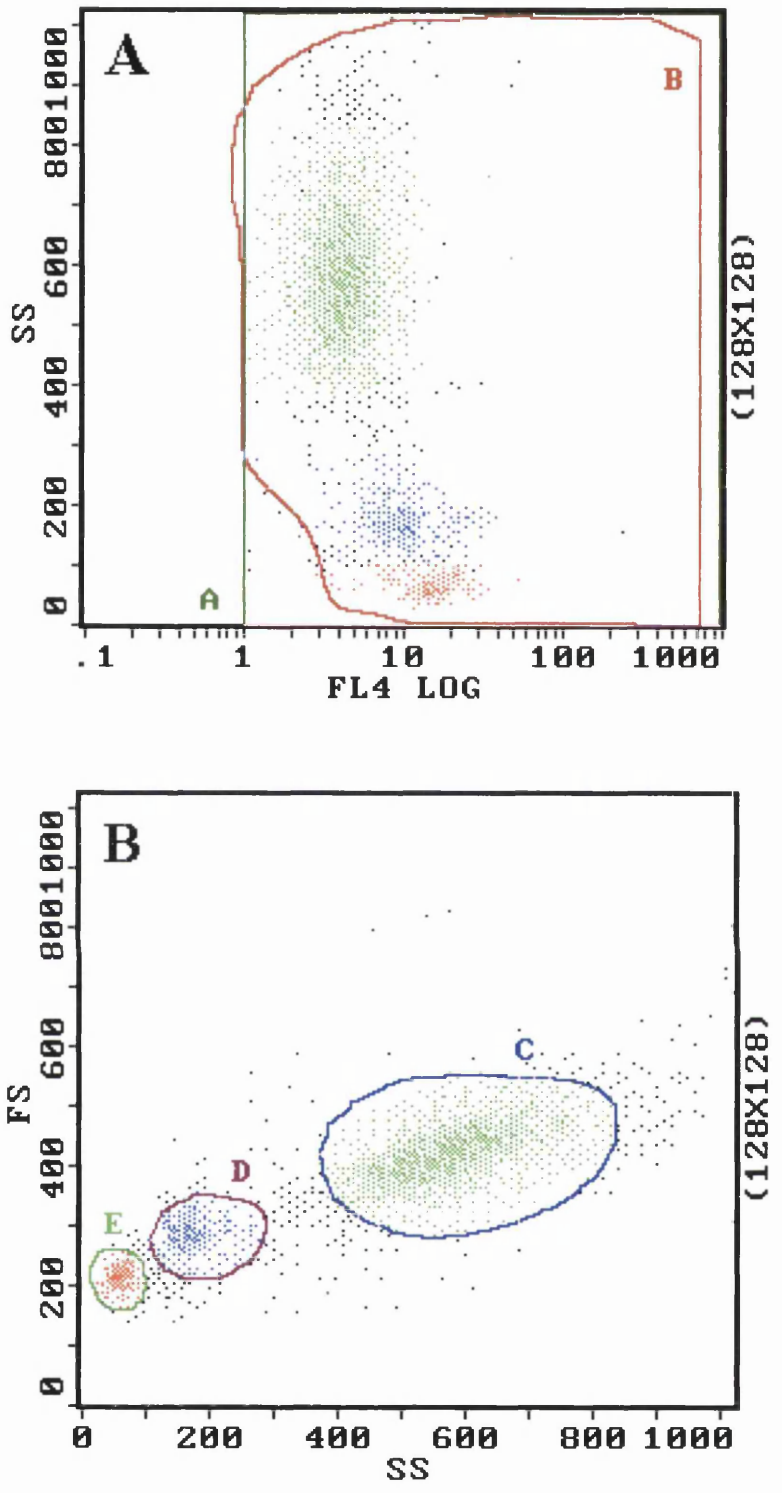

Figure 2.6 A and B. *Scatterplots demonstrating typical leukocyte scatter profiles. In Figure (A), only cells expressing the leukocyte antigen CD45 are analysed and these are detected on FL4. Figure (B) demonstrates the three leukocyte populations (gated from Figure (A)) of granulocytes (region 'C'), monocytes (region 'D') and lymphocytes (region 'E') which have distinct scatter profiles.*

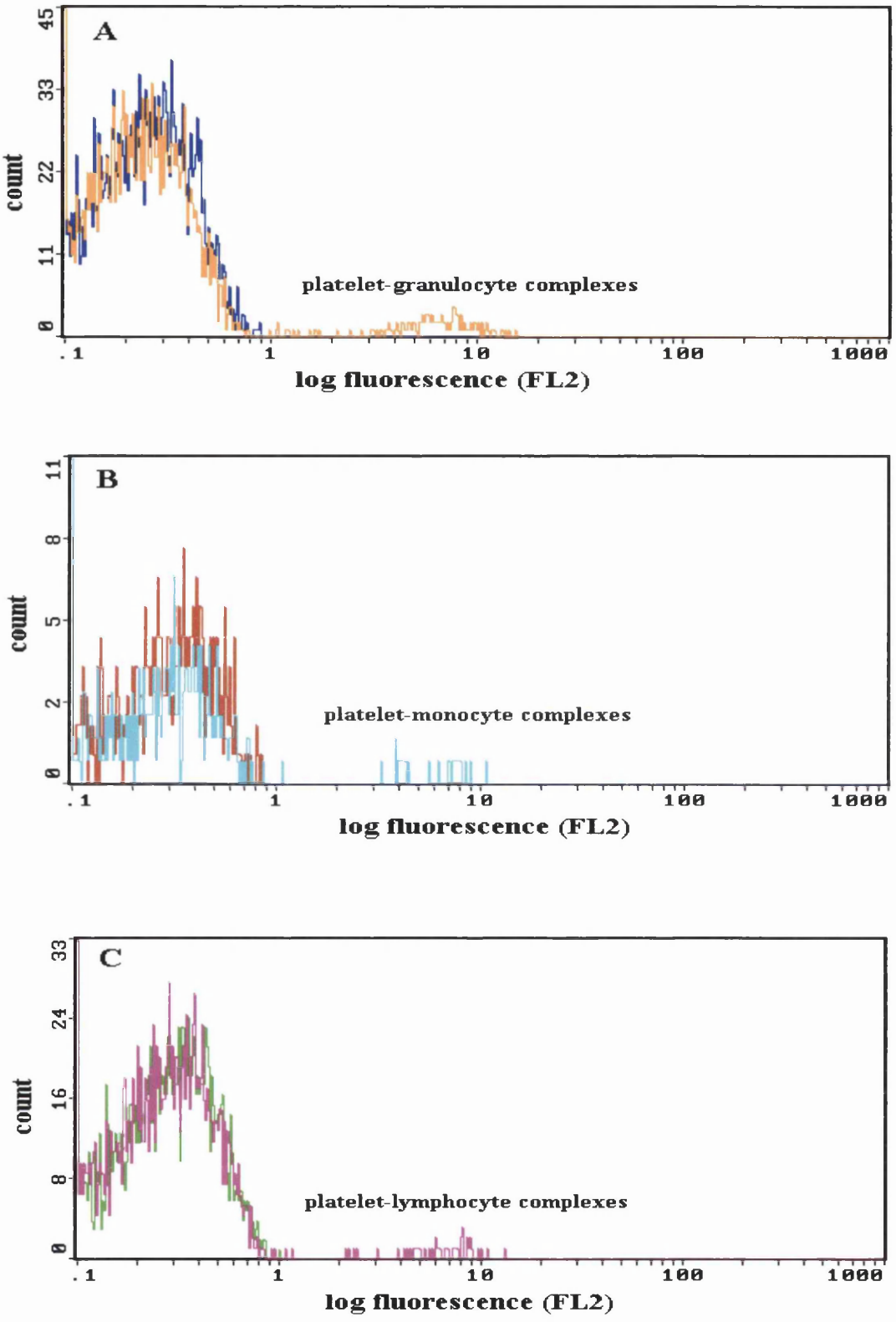

Figure 2.7 A, B and C *Histograms demonstrating typical fluorescence profiles for platelet-granulocyte (A), platelet-monocyte (B), and platelet-lymphocyte (C) complexes, where platelets are stained with CD42b-PE. Less than 5% of all circulating leukocytes are complexed with platelets. Count = cell number*

Method development

In setting up a method for the flow cytometric detection of platelet-leukocyte aggregates (PLA), a number of different approaches were attempted and the results of these experiments are summarised below.

1) Whole blood method without erythrocyte lysis -

In this experiment, 5 µl whole blood was added to 5 µl anti-CD42b and 5 µl anti-CD45 in HBS to make a total volume of 50 µl. After a 20 minute incubation at room temperature, the samples were further diluted in HBS before being analysed on the flow cytometer. Although only CD45 positive events were analysed, it was often difficult to adequately differentiate erythrocytes from leukocytes due to their much higher concentration in whole blood. Another limitation of this method was the very long analysis time required to collect an adequate number of events. Increasing the volume of blood used in the initial incubation did not result in any significant improvement, hence methods which involved erythrocyte lysis were examined.

2) Whole blood method with erythrocyte lysis (using 'Multi Q -prep' workstation) -

The routine flow cytometric analysis of leukocyte markers involves the use of the 'Multi Q-prep workstation' (Beckman Coulter), an instrument which allows the lysis and fixation of multiple samples. It utilises the Immunoprep reagent (Beckman Coulter), which is composed of reagent A (to lyse erythrocytes), reagent B (a buffer solution which stops the lysing process), and reagent C (which finally fixes cells). In the next set of experiments, this instrument and its reagents were used. 50 - 100 µl whole blood was incubated with 10 µl anti-CD42b and 10 µl anti-CD45 for 30 minutes at room temperature. Following this the samples were then placed on the 'Multi Q-prep' before being analysed on the flow cytometer. Due to the erythrocyte

lysis, there was no difficulty in identifying leukocytes, and adequate cell numbers were analysed in a relatively short period of time. Basal levels of circulating total PLA using this method varied from 7.7% to 26.7% in different resting normal individuals. The addition of the platelet agonist TRAP, resulted in a significant increase in total PLA as expected (in particular, platelet-monocyte aggregates), verifying the ability of the method to detect increased PLA. However, a number of problems with this method were noted. Firstly, the number of PLA decreased significantly following a delay in analysis after the 'Multi Q-prep' procedure. There was an approximate 50% decrease in PLA when samples were analysed immediately compared to a 40 minute delay post 'Multi Q-prep'. Another problem was a lack of reproducibility between results - the interassay coefficient of variation (CV) for this method was 30%. It was thought that the 'Multi Q-prep' procedure may be too vigorous and actually cause the formation of PLA, which may dissociate over time. This method was abandoned, and further ways of producing erythrocyte lysis without inducing PLA formation were investigated.

3) Whole blood method with erythrocyte lysis (using 'Optilyse ® C') -

In this method, whole blood was incubated with anti-CD42b and anti-CD45 monoclonal antibodies. 'Optilyse ® C' (Immunotech, Beckman Coulter) solution, an erythrocyte lysing reagent, was then added in order to cause red cell lysis, and samples were analysed on the flow cytometer. On average, total PLA as measured by this method resulted in a lower percentage (i.e. 3.7%) than that obtained by the 'Multi Q-prep' method (19.2%) on the same sample of blood. Although the 'Optilyse ® C' did not appear to induce PLA formation, it did not result in the expected maximal increase in PLA formation (in particular platelet-neutrophil aggregates) when platelets

were stimulated maximally with TRAP. As a result of this finding, I felt that it was inappropriate to continue with this method and the reagent as its sensitivity was questionable.

4) Whole blood method with erythrocyte lysis (using distilled water) -

Whilst attempting to develop a method for PLA detection, a paper was published which found increased monocyte-platelet aggregates in patients with stable coronary artery disease (Furman *et al*, 1998). The method employed by the authors used distilled water to induce erythrocyte lysis following a 10 minute fixation period. This method was adapted for use in our laboratory, however upon initial testing, very high basal levels of PLA were found in normal individuals. After performing formal time course experiments (similar to those undertaken with PAC-1 binding), it was found that a time delay between venepuncture and antibody staining resulted in a significant increase in total PLA. TRAP activation of platelets resulted in the expected maximal increase in PLA. The results of the time course experiment are shown in *Figure 2.8* and demonstrate that there is a time-dependent increase in PLA formation (particularly platelet-monocyte aggregates) which becomes apparent at approximately 15 minutes following venepuncture. Notably, a previous study which measured the percentage of platelet-neutrophil complexes in freshly collected blood at intervals of up to 60 minutes after incubation on the bench at room temperature, found that there was a significant increase in such complexes by 45 minutes (Peters *et al*, 1997).

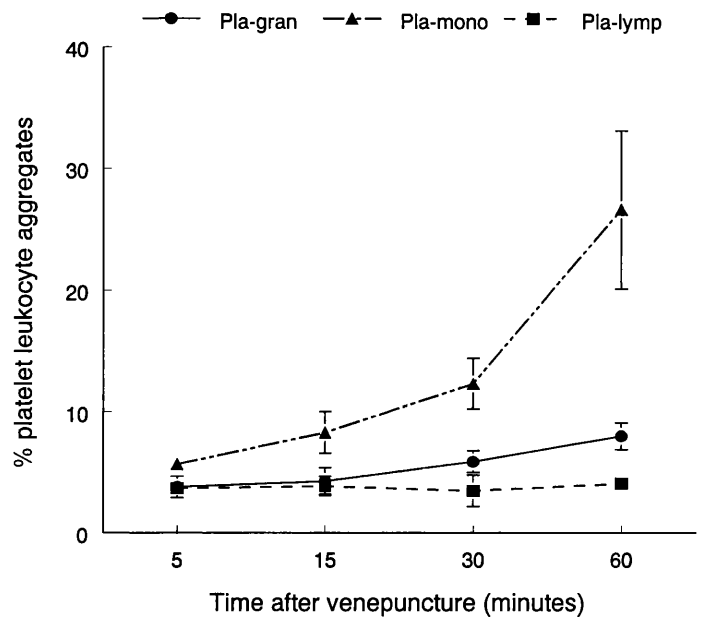

Figure 2.8 *Effect of time delay between venepuncture and sample antibody staining on % circulating platelet leukocyte aggregates (platelet-monocyte, platelet-granulocyte and platelet-lymphocyte) in controls (n=3).*
Mean ± SD are indicated.

2.2.7 Platelet-derived microparticles

Principle

Platelet-derived microparticles form when there is vesiculation of the platelet plasma membrane and result from activation of platelets by a number of agonists/during storage, or as a result of mechanical/shear forces (as in freeze/thaw or cardiopulmonary bypass). Their size can vary, however for the purposes of flow cytometry, some authors have defined them as < 1.0 μm (Horstman *et al*, 1994); and they generally express membrane GPIb, IIb and IIIa. These characteristics make it possible to identify and count microparticles using flow cytometry.

Reagents

- Monoclonal antibody anti-CD41-RPE (Dako)

- Isotype control IgG1-PE (Immunotech, Beckman Coulter)
- Isoton® II (Beckman Coulter)
- Latex Polystyrene Beads (Sigma-Aldrich) - 0.8 μm diameter

Final method

Platelet poor plasma (30 μl) was incubated with 5 μl monoclonal antibody anti-CD41-RPE/isotype control for 30 minutes at room temperature and then diluted in 0.5 ml filtered Isoton® II. Each tube was then weighed immediately before being analysed on the flow cytometer. The microparticle gate had been defined previously using Latex Polystyrene Beads (Sigma-Aldrich) beads of 0.8μm diameter (see 'Method Development'), and only GP IIb/IIIa positive events which fell within this region (i.e. < 0.8 μm) were classified as platelet microparticles. Each sample was analysed for 2 minutes and the flow rate was maintained on 'high'. The 'dead volume' was also calculated daily, by stopping analysis immediately after it had commenced. Following all sample analyses, the tubes were again weighed and the volume which had been measured was calculated according to a sample volume/weight ratio which had been previously calibrated for that particular sample type. Results were then expressed as the number of microparticles/ml plasma. The normal reference range for numbers of platelet microparticles was based on the geometric mean ± 2 SD of log normalised data from 20 normal individuals, and was 1.1×10^5 - 1.2×10^6 microparticles/ml plasma. The inter assay CV for measuring microparticles in resting samples was 2.7%.

Method validation

The ability of this method to detect platelet-derived microparticles was verified in control experiments using microparticles which had been generated from outdated

platelet concentrates which had been spun to obtain PPP, before being frozen, and on thawing, spun again (Horstman *et al*, 1994). The light scatter and fluorescent profile of generated platelet microparticles are shown in *Figure 2.9.*

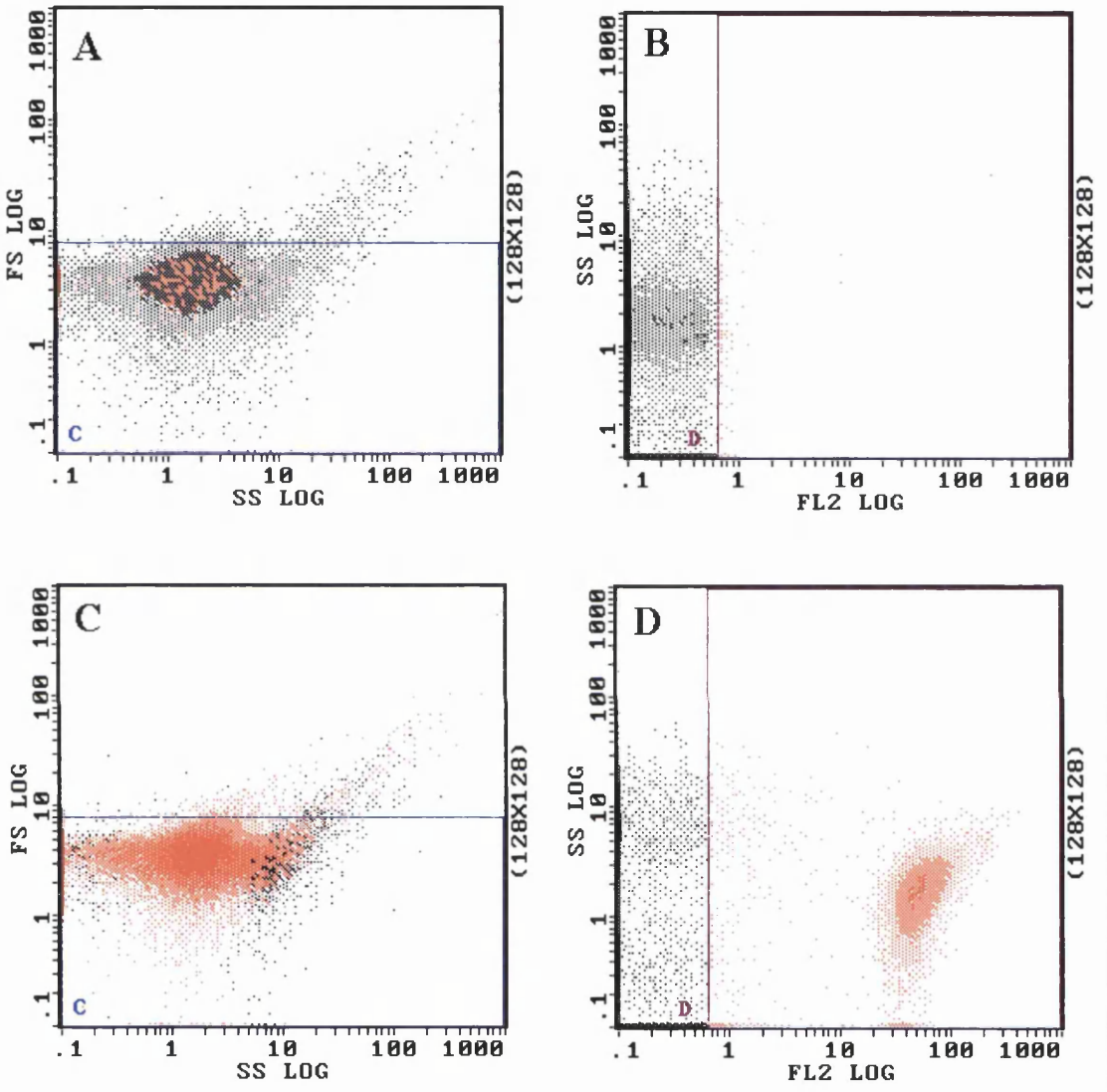

Figure 2.9 A, B, C and D *demonstrating histograms of generated platelet microparticles used as a positive control. Figure A and B are control histograms demonstrating light scatter and fluorescence profiles (respectively) of microparticles stained with IgG isotype control; figures C and D correspond to the same histograms of microparticles stained with anti-CD41-PE monoclonal antibody. The majority of microparticles fall within the gate which has been set according to 0.8 μm beads.*

Method development

In setting up a method for microparticle counting in our laboratory, the literature was reviewed, and it was noted that one of the difficult aspects of microparticle studies had been the lack of a reproducible quantitative assay. Problems with direct particle counting are that the minute size of some microparticles may lead to an underestimation of their number. Also, it is known that some microparticles may have weak/absent GPIb markers (Jy *et al*, 1992).

Whole blood methods have been used to measure microparticles. These methods distinguish microparticles from platelets by using a horizontal marker set at the lower limit of the platelet cloud to separate the two populations. One problem with this technique is that the platelet cloud scatter can vary from patient to patient, and so the marker must be set arbitrarily. Microparticles can also be counted in platelet poor plasma samples. A number of groups have applied this method, using calibrated beads to both define the microparticle gate as well as count numbers of microparticles. The method used in this thesis was based on one which had been recently published (Combes *et al*, 1997). In this paper, microparticles were first extracted from platelet poor plasma before being incubated with a PE-conjugated monoclonal antibody against GP IIb/IIIa. Prior to flow cytometric analysis, a definite number of Latex Polystyrene Beads (Sigma-Aldrich) of 3.0 μm size, were added to each tube. The microparticle gate had been defined previously using Latex Polystyrene Beads (Sigma-Aldrich) of 0.8 μm *(Figure 2.10),* and hence only GP IIb/IIIa positive events which fell within this region were classified as platelet microparticles.

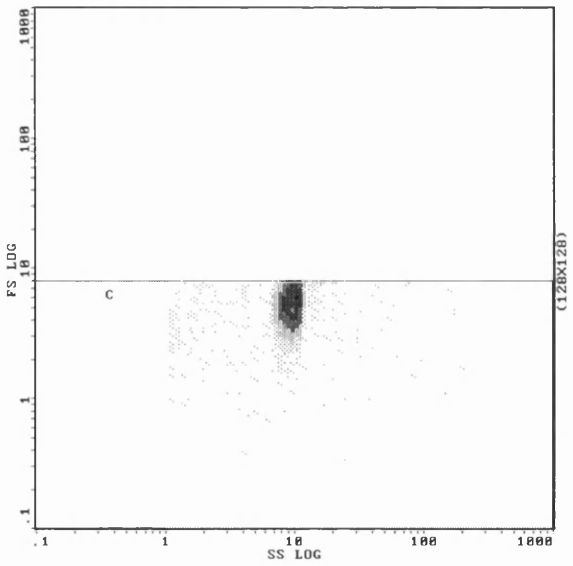

Figure 2.10 *A scatterplot of log forward versus side scatter demonstrating the light scatter characteristics of 0.8 μm beads. A gate (region 'C') can thus be drawn to identify particles of this size or smaller.*

The number of microparticles in 1 ml of plasma was calculated by counting a pre-determined bead number. The authors found that the plasma concentration of microparticles appeared to be independent of the platelet count. When applying this method, it was demonstrated that although the number of GP IIb/IIIa positive events could be accurately counted, the number of polysytrene beads (Sigma-Aldrich) added to the tube as an internal standard could not be accurately determined since there was some agglutination of the beads. This was unavoidable, and so it was impossible to give an accurate concentration of microparticles in plasma by using this method.

Following this, a series of experiments designed to determine whether or not the flow cytometer analysed a constant sample volume over a particular time period were performed. Samples of Isoton® II (Beckman Coulter) were analysed for fixed periods of time ranging from between 1 and 240 seconds. The volumes measured by the flow cytometer were calculated by weighing the sample tube pre and post analysis,

and calibrating a sample volume/weight ratio for each sample type. As can be seen from *Figure 2.11,* the flow cell does analyse volume at a fixed rate.

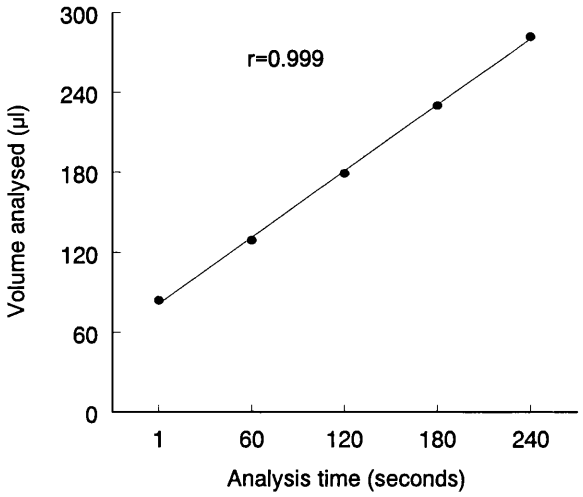

Figure 2.11 *Volume of sample analysed by Coulter® Epics® XL-MCL flow cytometer over varying time periods ranging between 1 and 240 seconds (n=5).* Mean ± SD are indicated

If using this method, one also has to determine the 'dead volume' of the flow cell, and take this into account when calculating the actual volume analysed. The dead volume was calculated by stopping the analysis immediately after it had commenced, and as can be seen from *Figure 2.12,* this remained fairly constant over time (checked on a daily basis). Fluctuations in dead volume were usually related to changes in the ambient temperature or a change in sample tubing.

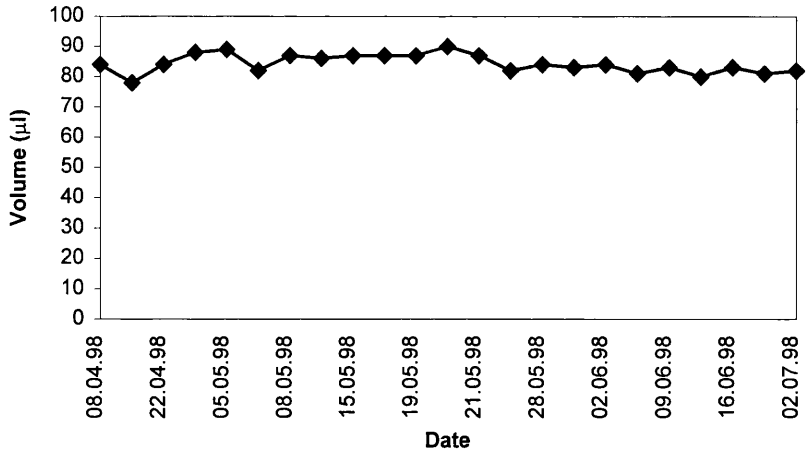

Figure 2.12 *Graph demonstrating fluctuations in dead volume of flow cytometer over a time period of three months.*

Recently, a method which uses a captured ELISA technique to measure microparticles has been published (Miyamoto *et al*, 1998). Platelet microparticle-sedimented material was obtained by ultracentrifuging (100,000 *g* for 2 hours) platelet free plasma. The sediment was then dissolved and applied to ELISA plates which were coated with the disintegrin, kistrin. The amount of GPIIb/IIIa antigen bound to kistrin is measured and expressed as the amount of plasma microparticles equivalent to purified GPIIb/IIIa complex. The authors of this paper feel that this method is more sensitive and less variable than flow cytometry or radioimmunoassay. For the purposes of this thesis however, a flow cytometric method to detect platelet microparticles was used.

2.2.8 Reticulated platelets

Principle

Reticulated platelets are 'young' platelets containing RNA. An increase in their number may indicate increased platelet turnover and shortened platelet survival. The

RNA fluorochrome thiazole orange (TO) can be used to identify platelets which contained some residual mRNA. The method used to measure reticulated platelets was that adapted by Robinson *et al*, 1998.

Reagents

- Thiazole Orange (Sigma-Aldrich) - a 1mg/ml stock solution of TO was made by dissolving the solid in methanol (Merck) and storing it in the dark at -20°C. A fresh working solution of 20 ng/ml TO was then prepared immediately prior to use.
- Isoton® II (Beckman Coulter)

Method

Whole blood (5 µl) was incubated with either 1ml of 20 ng/ml TO or Isoton® II which was used as a control. After a 30 minute incubation in the dark, the sample was than centrifuged at 1500 *g* for 3 minutes. The supernatant was discarded and the remaining pellet gently resuspended in 1 ml Isoton® II before being analysed on the flow cytometer. The normal reference range for percentage reticulated platelets was based on the geometric mean ± 2 SD of log normalised data from 12 normal individuals, and was 7.0% - 13.2%. The inter assay CV for measuring percentage reticulated platelets was 3.6%.

2.2.9 In vitro experiments

A series of experiments designed to determine the effects of purified IgG from patients with antiphospholipid syndrome on platelet activation were performed. These form the basis of Chapter 5, hence all methodology related to these experiments will be described at length in that chapter, together with the relevant findings.

2.3 DADE PFA-100™ Platelet function analyser

Principle

The DADE PFA-100™ platelet function analyser reproduces in-vitro and under high shear, the conditions to which a platelet would be exposed in an injured blood vessel. It allows evaluation of platelet function on small samples of citrate anticoagulated whole blood.

The test cartridge system consists of several integrated parts including a capillary, a sample reservoir and a biologically active membrane with a central aperture. By application of a constant vacuum, anticoagulated whole blood is aspirated from the sample reservoir through the capillary and the aperture under standardised conditions. The aperture simulates the injured part of a blood vessel - its membrane is coated with collagen and an agonist. Similar to the *in vivo* mechanism, platelets adhere and aggregate at the aperture thereby gradually diminishing and finally arresting the blood flow. The instrument determines the time from the start of the test until the platelet plug fully occludes the aperture, and reports that as the Closure Time (CT). This CT indicates the platelet function in the analysed blood sample.

There are 2 types of cartridge available for use. The Collagen/Epinephrine test cartridge is the primary cartridge used to detect platelet dysfunction. The Collagen/ADP test cartridge is used to indicate if an abnormal result obtained with Collagen/Epinephrine was caused by the effect of aspirin (ASA).

Quality Control

The PFA-100™ analyser has a self diagnostic test built in to verify proper instrument performance. This 'self test' was performed at the start of each day. Blood drawn

from healthy individuals (free from any medication known to affect platelet function), was used as normal controls.

Reagents

All reagents were supplied by Dade Behring and include:

- Dade®PFA Collagen/Epinephrine Test Cartridge - membrane coated with 2 μg equine Type I collagen and 10 μg epinephrine bitartrate
- Dade®PFA Collagen/ADP Test cartridge - membrane coated with 2 μg equine Type I collagen and 50 μg ADP
- Dade®PFA Trigger Solution - 0.9% aqueous sodium chloride

Method

This test used whole blood, collected into tubes containing 0.105M buffered sodium citrate (1 part anticoagulant to 9 parts blood). Test cartridges were warmed to room temperature prior to opening, before pipetting 800 μl blood into the smaller opening of the test cartridge(s), minimising air trapping. The cartridge(s) was loaded onto the instrument carousel, and the analysis started. The PFA-100™ platelet function analyser measured the Closure Time (see 'principle' above) in seconds, and a printout was provided. Examples of a printout from the PFA-100™ performed on a normal subject, and a patient with platelet dysfunction are shown in *Figure 2.13.* The normal closure time range established in our laboratory from 20 healthy controls is 61.4 - 104.7 seconds for Collagen/ADP cartridge and 78.9 - 139.3 seconds for Collagen/Epinephrine cartridge.

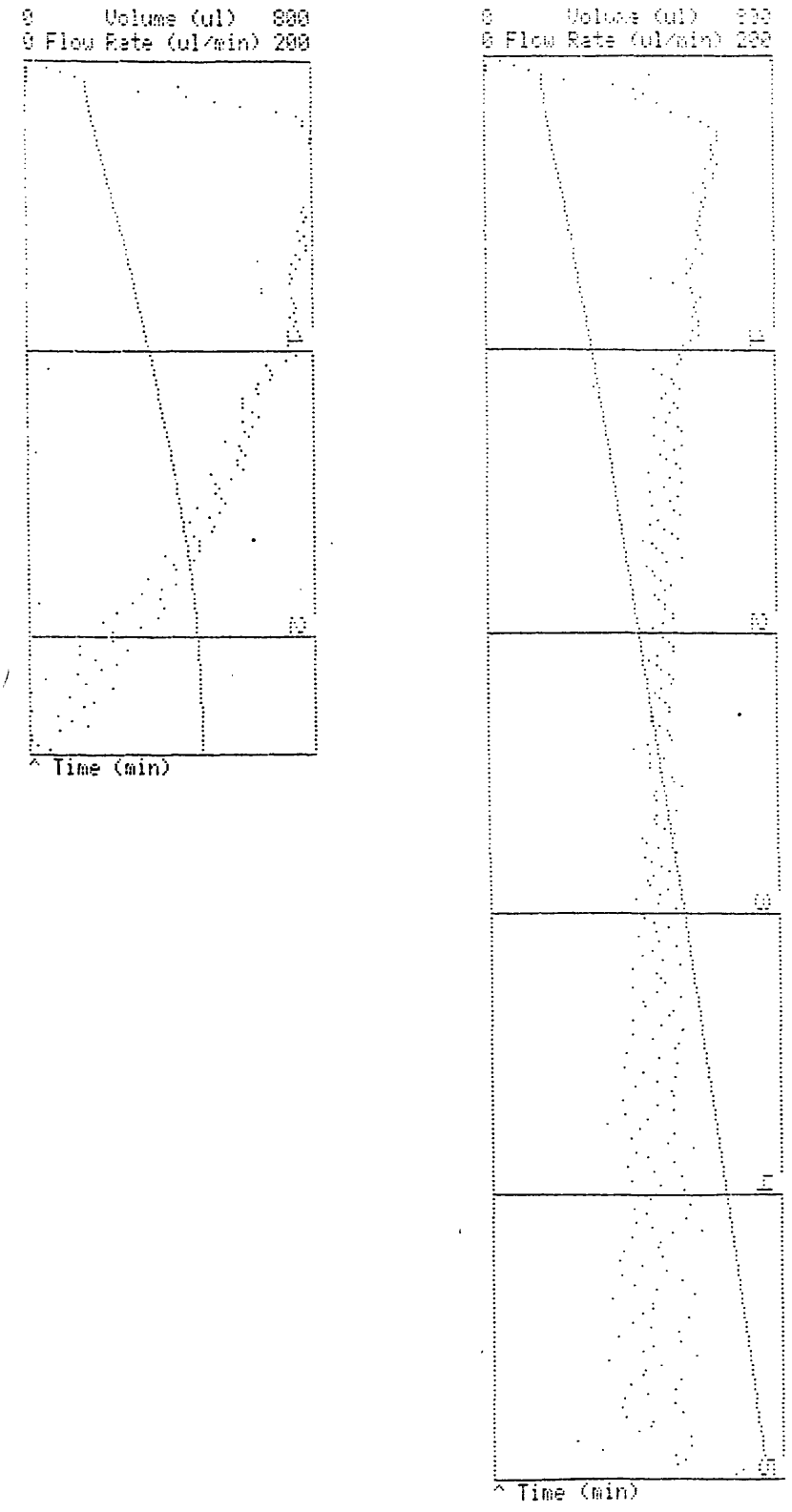

Figure 2.13 *Printouts from the PFA 100™ from a healthy control (left panel) and a patient with platelet dysfunction (right panel) using the Collagen/EPI cartridge.*

2.4 Enzyme Linked Immunosorbent Assays (ELISA)

2.4.1 General principle

The general principle of an ELISA is to bind antibody to the wells of a microtitre plate, expose it to antigen which is then 'captured' by antibody, and detect captured antigen by using either the same antibody (single determinant assay) or an antibody reactive to a different epitope of the antigen (double-determinant assay). The detecting antibody is coupled to an enzyme such as peroxidase, and any bound enzyme is then detected by placing substrate in the wells and developing a chromogenic product, the colour being measured by a spectrophotometer. By using suitable standards of known concentration, the procedure can be made quantitative, and so the amount of antigen present is proportional to the amount of colour produced.

2.4.2 Quality control

A quality control (QC) plasma was included in all ELISA assays performed. In most commercial ELISA kits, the QC plasma was provided. If not, then an 'in house' QC plasma was prepared from a pool of at least 20 healthy subjects. The reference range for this plasma was defined as the mean ± 2 SD from assays performed on at least 20 separate occasions. Results from assays where the QC plasma fell outside of the reference range were not accepted. All assays were performed in duplicate. If the coefficient of variation (CV) between duplicates was greater than 10%, the results were not accepted.

2.4.3 Thrombin-Antithrombin III (TAT) complex

Principle

The conversion of prothrombin into thrombin is a key event in coagulation, and the newly formed thrombin is then able to act on a number of substrates. It is also inhibited by antithrombin, which results in an inactive proteinase/inhibitor thrombin-antithrombin (TAT) complex, the concentration of which can be measured quantitatively by enzyme immuoassay.

Reagents

All reagents were supplied by Behring Ltd as part of a kit (Enzygnost® TAT micro), and include:

- 96 well microtitre plate coated with rabbit antibodies against human thrombin
- Rabbit anti-human ATIII, peroxidase conjugated
- Conjugate buffer - Tris buffer solution (50 mmol/l) and bovine serum albumin
- TAT standard plasmas - concentrations ranging between 2 to 60 μg/l
- TAT control plasma
- Sample buffer - Tris buffer solution (100 mmol/l), Tween (10ml/l) and EDTA (37g/l)
- Washing solution - PBS (90 mmol/l) containing Tween (18g/l)
- Buffer/substrate - Hydrogen peroxide (0.3g/l) in citrate buffer solution
- Chromogen - o-phenylenediamine dihydrochloride
- Stopping solution - 0.5 N sulphuric acid

Method

All determinations were performed in duplicate. Sample buffer (50μl) plus either standard, control or test plasma (50μl) were added to each well of the microtitre plate,

and after gentle mixing, incubated for 15 minutes at 37°C. Each well was then aspirated and washed three times with 300 µl of washing solution. 100 µl of conjugate solution was added and incubated for 15 minutes at 37°C. All wells were again aspirated and washed three times, before adding 100 µl of freshly prepared chromogen substrate. Plates were incubated for 30 minutes at room temperature (protected from light) and the reaction was stopped by adding 100 µl of stopping solution. Absorbance values were measured at a wavelength of 492 nm, using an automated plate reader. Mean absorbance values of the standards were used to plot the reference curve (log-log) from which concentrations of test samples were then derived. The within assay CV (intra-assay CV) for this method is 4 - 6%, and the between assay CV (inter-assay CV) is 6 - 9%. The kit reference range for levels of TAT was 1.0 - 4.1µg/l. In our laboratory, the reference range based on the geometric mean ± 2 SD of log normalised data from 20 normal individuals was 0.7 - 5.8 µg/l.

2.4.4 Prothrombin Fragment F 1+2 (F 1+2)

Principle

When prothrombin is converted into thrombin, there is also the formation of the prothrombin fragment F 1+2. Through the use of an enzyme immunoassay to detect F 1+2, it is possible to quantify exactly the actual amount of thrombin formed.

Reagents

All reagents were supplied by Behring Ltd as part of a kit (Enzygnost® F 1+2 micro), and include:

- 96 microtitre plate coated with rabbit antibodies to human F 1+2
- Rabbit anti-human prothrombin, peroxidase conjugated

- Conjugate buffer - Tris buffer solution containing Tween, bovine serum albumin
- F 1+2 standard plasmas - concentrations ranging between 0.04 to 10 nmol/l
- F 1+2 control plasma
- Sample buffer - Tris buffer solution containing Tween sodium chloride
- Washing solution - PBS (90 mmol/l) containing Tween (18g/l)
- Buffer/substrate - Hydrogen peroxide (0.3 g/l) in citrate-phosphate buffer solution
- Chromogen - o-phenylenediamine-dihydrochloride
- Stopping solution - 0.5 N sulphuric acid

Method

All determinations were performed in duplicate. Sample buffer (50µl) plus either standard, control or test plasma (50 µl) were added to each well of the microtitre plate, and after gentle mixing, incubated for 30 minutes at 37°C. Each well was then aspirated and washed three times with 300 µl of washing solution. 100 µl of conjugate solution was added and incubated for 15 minutes at 37°C. All wells were again aspirated and washed three times, before adding 100 µl of freshly prepared chromogen substrate. Plates were incubated for 15 minutes at room temperature (protected from light) and the reaction was stopped by adding 100 µl of stopping solution. Absorbance values were measured at a wavelength of 492 nm, using an automated plate reader. Mean absorbance values of the standards were used to plot the reference curve (log-log) from which concentrations of test samples were then derived. The intra-assay CV for this method is 5 - 7.5%, and the inter-assay CV is 6 - 13%. The kit reference range for levels of F 1+2 was 0.4 - 1.1 nmol/l. In our laboratory, the reference range based on the geometric mean ± 2 SD of log normalised data from 20 normal individuals was 0.3 - 1.8 nmol/l.

2.4.5 Soluble P-selectin

Principle

Soluble P-selectin is found in the plasma of normal individuals at ng/ml concentrations, and probably arises from both platelets and endothelial cells. Levels of soluble P-selectin may be elevated in a variety of pathological conditions, and can be measured by a quantitative sandwich immunoassay technique.

Reagents

All reagents were supplied by R&D Systems as part of a kit (Parameter® Human soluble P-selectin immunoassay), and include:

- 96 well microtitre plate coated with a murine monoclonal antibody to human sP-selectin
- Sheep anti-human sP-selectin, peroxidase conjugated
- Conjugate diluent
- sP-selectin standards - 6 vials of lyophilized recombinant soluble human P-selectin
- Parameter control - 1 vial of lyophilized human serum containing sP-selectin
- Sample diluent
- Wash buffer concentrate
- Substrate - tetremethylbenzidine
- Stop solution

Method

All determinations were performed in duplicate. Samples and control were diluted either 10-fold or 20-fold with sample diluent. 100 µl of standard, diluted sample or diluted control were added to each well of the microtitre plate. Next, 100 µl of diluted

anti-sP-selectin conjugate was added and wells were gently mixed. Plates were incubated for 1 hour at room temperature. Each well was then aspirated and washed three times with 300 µl of wash buffer, before adding 100µl of substrate. After a further 15 minute incubation at room temperature, the reaction was stopped by adding 100 µl of stop solution. Absorbance values were read at 450 nm with a wavelength correction set to either 620 or 650 nm, using an automated plate reader. Mean absorbance values of the standards were used to plot the reference curve (lin-lin) from which concentrations of test samples were then derived, remembering to multiply by the dilution factor. The intra-assay CV for this method is 4.9 - 5.6%, and the inter-assay CV is 7.9 - 9.9%. The kit reference range for levels of sP-selectin was 20 - 44 ng/ml. In our laboratory, the reference range based on the geometric mean ± 2 SD of log normalised data from 19 normal individuals was 6.9 - 52.4 ng/ml.

2.4.6 Soluble E-selectin

Principle

Soluble E-selectin is found in the blood of healthy individuals at ng/ml concentrations, and probably arises from proteolytic cleavage of the surface-expressed molecule which is found on endothelial cells following their activation by inflammatory cytokines. Levels of soluble E-selectin may be elevated in a variety of pathological conditions, and can be measured by a quantitative sandwich immunoassay technique.

Reagents

All reagents were supplied by R&D Systems as part of a kit (Parameter® Human soluble E-selectin immunoassay), and include:

- 96 well microtitre plate coated with a murine monoclonal antibody to human sE-selectin
- Anti-human sE-selectin, peroxidase conjugated
- Conjugate diluent
- sE-selectin standards - 6 vials of lyophilized recombinant human sE-selectin
- Parameter control - 1 vial of lyophilized human serum containing sE-selectin
- Sample diluent
- Wash buffer concentrate
- Substrate - tetremethylbenzidine
- Stop solution

Method

All determinations were performed in duplicate. Samples and control were diluted 20-fold with sample diluent. 100 µl of diluted anti sE-selectin conjugate was added to each well of the microtitre plate. Next, 100 µl of standard, diluted sample or diluted control were added and wells were gently mixed. Plates were incubated for 1.5 hours at room temperature. Each well was then aspirated and washed six times with 300 µl of wash buffer before adding 100 µl of substrate. After a further 30 minute incubation at room temperature, the reaction was stopped by adding 100 µl of stop solution. Absorbance values were read at 450 nm with a wavelength correction set to either 620 or 650 nm, using an automated plate reader. Mean absorbance values of the standards were used to plot the reference curve (lin-lin) from which concentrations of test samples were then derived, remembering to multiply by the dilution factor. The intra-assay CV for this method is 4.7 - 5%, and the inter-assay CV is 5.7 - 7.4%. The kit reference range for levels of sE-selectin 29.1 - 63.4 ng/ml. In our laboratory, the

reference range based on the geometric mean ± 2 SD of log normalised data from 30 normal individuals was 16.5 - 67.4 ng/ml.

2.4.7 Thrombomodulin

Principle

Thrombomodulin (TM) is a glycoprotein which converts thrombin from a procoagulant protease to an anticoagulant. It is found on the surface of endothelial cells of arteries, veins, capillaries and lymphatics, and increased levels of TM reflect injury of the endothelial cells, such as may occur in DIC. Levels of TM in plasma can be measured by a quantitative sandwich immunoassay technique.

Reagents

All reagents (apart from 3 M sulphuric acid) were supplied by Diagnostica Stago as part of a kit (Asserachrom Thrombomodulin Enzyme Immunoassay of Thrombomodulin), and include:

- 96 well microtitre plate coated with the F(ab')2 fragments of an anti-thrombomodulin monoclonal antibody
- Anti-thrombomodulin, peroxidase conjugated
- Dilution buffer - containing phosphate, bovine albumin, and tween 20
- Reference thrombomodulin
- Calibration diluent - diluted thrombomodulin deficient plasma
- Washing solution
- Substrate - ortho-phenylenediamine (OPD), which is diluted with urea peroxide tablets and distilled water immediately prior to use
- 3 M sulphuric acid - used as the stop solution

Method

All determination were performed in duplicate. The reconstituted reference thrombomodulin was diluted serially (1:2, 1:4, 1:8 and 1:16) with calibration diluent to prepare the assay calibrators. Samples were diluted either 5-fold or 10-fold with dilution buffer. 200 µl of diluted sample or calibrator were added to each well of the microtitre plate and wells were gently mixed. Plates were incubated for 2 hours at room temperature. Each well was then aspirated and washed five times with 300 µl of washing solution, before adding 200 µl of conjugate. After a further 2 hours incubation at room temperature, wells were again aspirated and washed five times with 300 µl of washing solution. Next, 200 µl of OPD/urea peroxide substrate was added to each well. After an 8 minute incubation at room temperature, the reaction was stopped by adding 50 µl of 3 M sulphuric acid to each well. After a further 10 minutes, absorbance values were read at 492 nm, using an automated plate reader. Mean absorbance values of the calibrators were used to plot the reference curve (log-log) from which concentrations of test samples were then derived, remembering to multiply by the dilution factor. The intra assay CV for this method is 4.2%, and the inter assay CV is 6.7%. In our laboratory, the reference range based on the geometric mean ± 2 SD of log normalised data from 34 individuals was 24.1 - 87.7 ng/ml.

2.4.8 D-dimer

Principle

The stabilised fibrin network which is formed during coagulation, is immediately degraded by the fibrinolytic enzyme, plasmin. A variety of crosslinked fibrin degradation products are formed during fibrinolysis, and the smallest of these fragments is D-dimer. Hence the detection of D-dimer indicates that the sequence of

thrombin activation, clot formation and subsequent clot lysis has occurred. D-dimer levels can be quantitated using an immunoassay, and elevated levels of occur in numerous conditions.

Reagents

All reagents were supplied by Quadratech as part of a kit (Agen Dimertest® Gold Stripwell EIA kit), and include:

- 96 well microtitre plate coated with monoclonal anti D-dimer antibody, DD-3B6
- Mouse monoclonal anti-FDP, peroxidase conjugated
- D-dimer standard - lyophilized high molecular weight D-dimer (2000 ng/ml)
- D-dimer control - lyophilized high molecular weight D-dimer (500 ng/ml)
- Tween 20
- Substrate - 2,2'-Azino-bis (3-Ethylbenzthiazoline Sulphonic Acid) (ABTS), which is activated with diluted 3% hydrogen peroxide immediately before use
- Stopping reagent
- Buffer - phosphate buffered saline

Method

All determinations were performed in duplicate. The reconstituted D-dimer standard was diluted serially (1:2, 1:4, 1:8, 1:16, 1:32 and 1:64) with diluent to prepare the assay calibrators. The microtitre plate was first washed (to remove azide) before adding 100µl of buffer to each well. Next, 25 µl of each D-dimer standard, sample or control were added. The plates were incubated at room temperature (with continual mixing) for 15 minutes. Each well was then aspirated and washed three times with 300 µl of wash buffer before adding 50 µl of reconstituted tag antibody to each well. After a further 15 minute incubation at room temperature (with continual mixing), the

wells were again aspirated and washed three times with buffer. Next, 100µl of activated ABTS substrate was added to each well. After a 15 minute incubation at room temperature (with continual mixing), the reaction was stopped by adding 50 µl of stopping reagent to each well. Absorbance values were read at 405 - 420 nm using an automated plate reader. Mean absorbance values of the calibrators were used to plot the reference curve (lin-lin) from which concentrations of test samples were then derived. The intra-assay CV for this method is 3.7 - 6.8%, and the inter-assay CV is 6.6 - 10.1%. The kit reference range for levels of D-dimer was < 120 ng/ml.

2.4.9 Anticardiolipin antibody

Principle

Microtitre plates are coated with purified cardiolipin. Test serum samples (diluted in adult bovine serum) are applied, and specific antibody binding to the cardiolipin is detected using a conjugated anti-human IgG or IgM. Thus both IgG and IgM anticardiolipin antibodies (ACL) may be detected. The assay was standardised using local reference sera cross calibrated against reference sera (a gift from R Malia, Royal Hallmshire Hospital, Sheffield) which in turn had been calibrated against the original Harris standards at an international workshop.

Reagents

All reagents were from Merck Ltd. unless otherwise stated, and include:

- Cardiolipin from bovine heart (ethanol solution, Sigma-Aldrich Chemical Co.)
- Ethanol - absolute alcohol
- Phosphate buffered saline (PBS): 0.01 M phosphate, 0.145 M sodium chloride adjusted to pH 7.2

- Adult bovine serum (ABS): 10% (v/v) ABS (Sigma-Aldrich Chemical Co.) in PBS
- Alkaline phosphatase conjugated goat anti-human IgG (γ chain specific) or IgM (μ chain specific) (Sigma-Aldrich Chemical Co.)
- 0.92 M diethanolamine buffer: pH 9.8, containing 0.5mM magnesium chloride hexahydrate
- Substrate: 1 mg/ml *p*-nitrophenyl phosphate disodium hexahydrate phosphatase (Sigma-Aldrich Chemical Co.) in diethanolamine buffer
- 3 M sodium hydroxide

Method

30 μl of a 50μg/ml solution of cardiolipin diluted in ethanol was added to the wells in rows C, D, E, F of a microtitre plate (Nunc Polysorp, Life Technologies Ltd.). 30 μl of ethanol was added to the wells in the remaining rows (blank wells). The plate contents were allowed to evaporate by being left uncovered overnight at 4°C and then washed with PBS. To reduce non-specific binding, plates were blocked with 75 μl of ABS buffer for 1 hour at room temperature. After washing once in PBS, 50 μl aliquots of doubling dilutions of reference serum (1/50 to 1/1600) and 1/50 dilutions of test samples in ABS buffer were added to both cardiolipin coated and blank wells in duplicate, which were then incubated at room temperature for 3 hours. After washing three times, 50 μl aliquots of alkaline phosphatase conjugated goat anti anti-human IgG or IgM diluted 1/100 in ABS buffer was added to each well and incubated at room temperature for 90 minutes. After washing, 50 μl aliquots of 1 mg/ml substrate (*p*-nitrophenyl phosphate disodium hexahydrate phosphatase) in diethanolamine buffer were added to each well and incubated in the dark at 37°C until a suitable level

of colour had developed. The reaction was stopped with 50 µl of 3 M NaOH. Absorbance values were read at 410 nm using an automated plate reader. The mean absorbance of the blank wells was initially subtracted from that of the test wells. Absorbance values of the standards were used to plot the reference curve (log-log) from which concentrations of test samples were then derived. The normal reference range for both IgG and IgM ACL was < 5 GPL or MPL units repectively.

2.4.10 Anti Beta$_2$glycoprotein I antibody

Principle

A quantitative indirect ELISA on high binding γ-irradiated microtitre plates using human β_2GPI to capture and immobilise autoantibodies is used. Immobilised antibodies are bound by a conjugated monoclonal anti-human IgG, the presence of which was revealed and quantified when exposed to a suitable substrate.

Method

A partly commercialised method was used, which had been validated by Ms S Donohoe (Haemostasis Research Unit, University College London).

Reagents

All reagents were supplied by Shield Diagnostic Ltd as part of a kit (Diastat Anti-Beta$_2$ Glycoprotein I) unless stated otherwise and include:

- Microtitre plate pre-coated with human β_2GPI
- Alkaline phosphatase conjugated murine monoclonal anti-human IgG in Tris buffer
- Wash buffer concentrate
- Sample diluent concentrate

- anti-β_2*GPI* positive control
- Substrate solution (phenolphthalein monophosphate)
- Stop reagent (carbonate buffer containing sodium hydroxide and EDTA)
- An 'in house' anti-β_2GPI standard from a well characterised antiphospholipid syndrome patient

Method

A range (1.56 to 100% activity) of standard solutions were prepared by doubling dilution buffer. Test and control samples were diluted 1 in 100 in dilution buffer. 100 µl of standards, tests or control were then added to the microtitre plate in duplicate within a time span of 4 minutes (limiting drift in results over plate positions) and incubated at room temperature for 1 hour. Unbound sample was removed by washing three times with 200 µl of washing solution. 100 µl of conjugated antibody was added and incubated at room temperature for 30 minutes. After washing a further three times, 100 µl of substrate solution was added to wells and incubated at room temperature for 30 minutes. The reaction was stopped and developed by the addition of 100 µl of stop reagent. Absorbance values were read at 550nm using an automated plate reader. Mean absorbance values of the standards were used to plot the reference curve (log-log) from which concentrations of test samples were then derived. The cut-off point for positivity (> 2.88%) was established as 2 SD above the mean on log transformed data of 30 healthy subjects. A plasma with known anti-β_2GPI concentration was assayed on each occasion to ensure reliability of the assay.

2.5 Routine haematology investigations

2.5.1 Full blood count (FBC)

A full blood count was performed on EDTA blood samples from all patients, using a Coulter STKS haematology analyser.

2.5.2 Erythrocyte Sedimentation Rate (ESR)

An erythrocyte sedimentation rate was performed on citrated blood samples (ratio of anticoagulant:blood was 1:4) from patients with SLE, using a Becton Dickinson Sediscan automated system.

2.6 Routine coagulation assays

All routine coagulation assays were performed on citrated plasma samples using either CA-1000 or CA-5000 coagulometers (Sysmex)

2.6.1 Prothrombin time (PT) and International Normalised Ratio (INR)

The prothrombin time was performed using Innovin (Dade/Sysmex UK Ltd), a recombinant human thromboplastin containing calcium chloride. The normal range is 10 - 12.5 seconds. The International Normalised Ratio (INR) was calculated according to the formula:

INR = (Test PT result/Control PT result)^{ISI} where ISI is the International Sensitivity Index of the thromboplastin which has been derived by calibrating the thromboplastin against an international reference preparation. The normal range for INR is < 1.3.

2.6.2 Activated Partial Thromboplastin Time (APTT)

The activated partial thromboplastin time was performed using Pathromtin SL (Dade Behring Ltd), an APTT reagent comprising silicon dioxide particles and vegetable phospholipids. The normal range is 22 - 36 seconds. All prolonged APTT's (i.e. those greater then 36 seconds), had 50:50 mixing tests, whereby APTT testing of a 50:50 mixture of normal control and patients plasma was performed. If the APTT of the mixture was closer to the patient's time than to that of the control, then the presence of an inhibitor is suggested.

2.6.3 Thrombin Time (TT)

The thrombin time was performed using Thromboclotin (Dade), after dilution of the bovine thrombin with saline to approximately 10 U/ml. The normal range is control ± 2 seconds.

2.6.4 Fibrinogen

Fibrinogen assays were performed with Dade Thrombin and Immuno Reference Plasma 100% (Technoclone Ltd) using the Clauss method whereby various dilutions of standard plasma and a dilution of test plasma are clotted with an excess of thrombin. The relationship between fibrinogen level and clotting time is linear over a certain range of concentrations, and therefore this factor can be assayed with a high degree of accuracy. The normal range is 1.5 - 4.5 g/l.

2.7 Lupus anticoagulant testing

A number of tests may be used to detect lupus anticoagulants (LA). For the purposes of this thesis, I will describe only those tests which were undertaken by our routine coagulation laboratory for LA detection in this particular study.

2.7.1 Dilute Russell's Viper Venom Time (DRVVT)

Principle

The DRVVT may be used for the detection of LA which block coagulant active phospholipid. Russell's Viper venom (RVV), phospholipid and calcium ions are added to plasma. RVV activates Factor X, and the resultant Factor Xa causes thrombin generation and fibrin clot formation. LA prolongs the clotting time, but it is corrected if the phospholipid reagent is replaced by washed, activated platelets. The latter are resistant to the action of LA. Dilution of RVV and phospholipid make it sensitive to LA.

Reagents

All reagents were supplied by Rho Reagents as part of a commerical kit (Unicorn Diagnostics Ltd Lupus Anticoagulant Kit) and include:

- Russell's Viper venom - reconstituted with sterile water
- Phospholipid reagent - reconstituted with sterile water
- Calcium chloride 0.05M - diluted 1 in 2 with distilled water to give a 0.025M calcium chloride solution
- Platelet neutralising reagent - lyophilised washed human platelets reconstituted with sterile water
- Pooled normal plasma (in house)

Method

The DRVVT was performed on the CA-1000/CA-5000. Clotting times were performed on sample and control plasmas using both phospholipid reagent and platelet neutralising reagent. A ratio of patient/control clotting times was calculated for both phospholipid reagent (PLR) and platelet neutralising reagent (PNR). Patients were defined as having LA if the PLR ratio of patient/control was > 1.10, and this was either corrected into the normal range or reduced by more than 10% in the platelet neutralisation procedure. Marked deficiencies of factors II, V, X or fibrinogen, or treatment with oral anticoagulants prolonged clotting times with both phospholipid reagent and platelet neutralising reagent, and this made identification of LA difficult. In these cases, testing an equal volume mixture ('50:50') of patient and control plasmas helps identification, since normal plasma will not correct ratios caused by LA, whereas prolonged clotting times due to factor deficiency should be completely corrected. In this study, many patients were receiving oral anticoagulant therapy which could not be discontinued. Thus, LA testing was performed on patient plasma alone, as well as a patient/normal mixture. All anticoagulated patients also underwent further LA testing with a Taipan Snake venom time.

2.7.2 Taipan Snake Venom Time (TSVT)

Principle

The Taipan Snake venom is able to convert prothrombin directly to thrombin in the absence of any of the other known clotting factors. Its action is independent of the plasma concentrations of factors V, X and VII. LA prolongs the clotting time, but it is corrected if the phospholipid reagent is replaced by washed, activated platelets. The latter are resistant to the action of LA. Dilution of TSV and phospholipid make it

sensitive to LA. The TSVT is primarily used to identify/confirm LA in patients on oral anticoagulants where the DRVVT may be negative or equivocal.

Reagents

- Imidazole buffer (0.5M) - 3.4g Imidazole, 5.48g NaCL. Add 900 ml distilled water and adjust pH to 7.3 before making to total volume 1L with distilled water.
- 1% albumin buffer. Dissolve 1g albumin in 100ml imidazole buffer, and store in aliquots at -70°C.
- Taipan Snake venom and $CaCL_2$ (TSV) (Diagnostic Reagents Ltd) - reconstituted according to the manufacturers instructions and then diluted approximately 1:3 with 1% albumin buffer.
- Phospholipid reagent ('Bell and Alton' Platelet Substitute, Diagnostic Reagents Ltd) - reconstituted in 5ml distilled water and diluted 1 part in 3 parts imidazole buffer. Stored in aliquots at -70°C.
- Calcium chloride 0.025M
- Platelet neutralising reagent (Unicorn Diagnostics/Rho reagents) - lyophilised washed human platelets reconstituted with sterile water

Method

The TSVT was performed on the KC4 (Amelung/Brownes Ltd). Clotting times were performed on sample and control plasmas using both phospholipid reagent and platelet neutralising reagent. A ratio of patient/control clotting times was calculated for both phospholipid reagent (PLR) and platelet neutralising reagent (PNR). Patients were defined as having LA if the PLR ratio of patient/control was > 1.10, and this was either corrected into the normal range or reduced by more than 10% with the platelet neutralising reagent.

2.8 Chromogenic coagulation factor assays

2.8.1 Factor VII assay

Principle

The reactions on which this assay are based occur in two stages. In the first stage, factor VII is activated by recombinant TF in the presence of calcium ions. This factor VIIa complex subsequently activates factor X. In the second stage, factor Xa causes the proteolytic cleavage of para-nitoaniline (pNA) from the chromogenic substrate S2765™. Liberation of the chromophore pNA is monitored kinetically at 405 nm. Within the linear range of the assay, the amount of factor Xa generated is directly proportional to the factor VII activity in the sample.

Reagents

- Owren's veronal buffer (Sysmex UK)
- 1.0M calcium chloride - dilute to 0.025M with distilled water
- Thromboplastin - Innovin™ (Sysmex UK) - diluted 1 in 5 in 25mM $CaCL_2$
- Factor X from human plasma (Sigma-Aldrich) - reconstituted with 1 ml of distilled water, and then diluted to 1 U/ml with Owren's veronal buffer.
- 25 mg chromogenic substrate S2765™ (Chromogenix) - diluted to 2mM with 17.49 ml distilled water
- Reference plasma 100% (Immuno Ltd) - reconstituted with 1 ml distilled water.

Method

The assay was performed on the CA-6000™ (Sysmex) and samples were analysed in duplicate at several dilutions. Factor VII activity was calculated according to the

principle that the amount of factor Xa generated is directly proportional to the factor VII activity in the sample.

2.8.2 Factor X assay

Principle

The reactions on which this assay are based occur in two stages. In the first stage factor X is activated by Russell's Viper venom in the presence of calcium ions. In the second stage, factor Xa causes the proteolytic cleavage of para-nitoaniline (pNA) from the chromogenic substrate S2765™. Liberation of the chromophore pNA is monitored kinetically at 405 nm. Within the linear range of the assay, the amount of factor Xa generated is directly proportional to the factor X activity in the sample.

Reagents

- Tris 50mM, NaCL 227mM buffer pH 8.3
- 1.0M calcium chloride - diluted to 0.40M with distilled water
- 0.2 mg/ml Russell's Viper Venom (Diagnostic Reagents Ltd) - reconstituted with 1 ml distilled water
- RVV and $CaCL_2$ solution - two volumes of 0.2 mg/ml RVV are mixed with one volume of 0.40M $CaCL_2$
- 25 mg chromogenic substrate S2765™ (Chromogenix) - diluted to 2mM with 17.49 ml distilled water
- Reference plasma 100% (Immuno Ltd) - reconstituted with 1 ml distilled water.

Method

The assay was performed on the CA-6000™ (Sysmex) and samples were analysed in duplicate at several dilutions. Factor X activity was calculated according to the

principle that the amount of factor Xa generated is directly proportional to the factor X activity in the sample.

2.9 Molecular biology tests

2.9.1 General

The **polymerase chain reaction (PCR)** is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the exponential amplification of a specific fragment whose termini are defined by the 5' ends of the primers.

Following is a description of the equipment and reagents necessary for PCR, as well as the method used to prepare the DNA, and a general PCR protocol. A brief description of each specific PCR test performed is then given.

Equipment

- DNA thermal cycler (Perkin Elmer)
- Gibco BRL horizontal gel electrophoresis apparatus (Life Technologies)
- Gibco gel casting system (Life Technologies)
- Electrophoresis power supply (Pharmacia)
- 1.5 ml eppendorf tubes (Perkin Elmer)
- 0.5 ml eppendorf tubes (Sarstedt)
- Microfuge II (Beckman)

- Blocked tips (Alpha)
- Transilluminator (UVP)
- Face shield

Reagents

- Ammonium chloride 0.091 g per 10 ml (Merck BDH)
- Sodium hyroxide 0.05M (Merck BDH)
- Tris 1M pH 7.5 - 8.0
- Enzyme (Mnl 1) (Bio Laboratories)
- 10X TBE (Tris Borate EDTA buffer) (Severn Biotech Ltd)
- 10X NTP's: made up from ATP, CTPc, TTP and GTP to give a final stock concentration of 2mM (Boehringer Mannheim)
- 10X Primers (50 mg each in a 25µl reaction)
- Oligonucleotides (Genosys)
- Enzyme - Red hot DNA polymerase (Advanced Biotechnologies Ltd)
- Ethidium Bromide 10mg/ml
- Sterile saline
- Sterile distilled water
- Mineral oil (Sigma-Aldrich)
- Agarose (Seakem LE) FMC Bio Products
- Xylene Cynole FF (Sigma X4126) 0.25%

DNA 'quick prep' method

For all work involving DNA preparation and PCR, gloves were always worn in order to avoid contamination with DNAase which is present on the skin. Initially, 100 µl of well mixed whole blood was pipetted into a 1.5 ml eppendorf tube using blocked tips

to avoid contamination. Then 800 µl of ammonium chloride solution (0.091g per 10 ml) was added, and mixed by inversion for 20 minutes. The samples were then centrifuged for two minutes in a microfuge. The supernatant was then removed and discarded, after first taking into solution the haemoglobin layer. The pellet was washed with 1 ml of 0.85% saline, and the sample again centrifuged for a further two minutes. The supernatant saline was removed and discarded. Next, 200 µl of 0.05M NaOH was added and the samples were vortexed. Samples were then heated to 99°C for 10 minutes on the thermal cycler. Following this they were neutralised by adding 40 µl of 1M Tris pH 7.5 - 8.0. Samples were again vortexed before being frozen and stored at -20°C.

General PCR protocol

The next stage involves making a 'master mix' consisting of nucleotides, specific primers (depending on which part of the DNA molecule is to be amplified), polymerase enzyme, and DNA sample for the PCR. For each run, the following volumes of reagents and patient 'Quick prep' sample were used:

Sterile water	7.5µl
10X buffer	2.5µl
10X NTPs	2.5µl
10X primers	2.5µl
Enzyme	0.1µl
Sample	10µl
Total	**25µl**

Although the primers and NTP's were quite stable, once thawed they were stored on ice. The reagents and sample were aliquoted into a small eppendorf tube. Two drops of mineral oil were then added to each tube in order to prevent evaporation of the sample during the PCR cycles. The sample tubes were placed on the thermal cycler,

and cycles of heating and cooling to various temperatures for several hours then took place.

Digestion

In the next stage, a digest consisting of 9 µl distilled water, 1µl 10X NEB buffer2 and 1U enzyme (Mnl 1) per sample was made up. 10µl of this digest was added to 10 µl of the PCR product (from the previous step) and incubated at 37°C overnight.

Preparation of agarose gel and gel set up

A 2% agarose gel (Factor V Leiden) and 3% agarose gel (Prothrombin gene mutation) were prepared by adding 100 ml of 1X TBE to 2g or 3g of agarose. After heating, ethidium bromide (10mg/ml) was added and the mixture poured into a mould to set. After setting, the gel was ready to be loaded and immersed in buffer in the electrophoresis tank. To 10µl sample, 3µl xylene dye was added. 12µl of this mixture was loaded carefully into each well in the gel. For Factor V Leiden detection, gels were run for 60 minutes at 100 V. For prothrombin gene mutation detection, gels were run for 120 minutes at 100V.

Results

The gel was then removed from the tank, and viewed on the transilluminator (UVP). A photograph was taken with a polaroid camera and results interpreted depending on the positions of bands on the gels of relevant controls.

2.9.2 Factor V Leiden

This genetic mutation involves the factor V gene and results in the replacement of Arg by Gln at position 506. The mutant factor V molecule has normal procoagulant activity, but is resistant to proteolysis by activated protein C. This variant is present in

approximately 4% of Caucasian factor V alleles, and is strongly associated with venous thromboembolism.

2.9.3 Prothrombin gene G20210A mutation

A point mutation in the prothrombin gene (G:A at position 20210) has been found to be over-represented in subjects with familial thrombosis. It is present in approximately 2% of healthy subjects, and the genotype is associated with an increased concentration of prothrombin in plasma.

2.10 Statistical analysis

Statistical analysis was performed using Astute software. The exact nature of the statistical analysis of data was dependent upon the number of observations and the distribution of the data obtained. Normality was assessed by examining histograms and skewness of data. In the majority of circumstances, data was distributed non-parametrically in either one/both of the control and patient groups, hence non-parametric tests (i.e. Wilcoxon Mann Whitney U and Spearman Rank Correlation Coefficient) were performed. A probability value of less than 0.05 was taken as being statistically significant.

3. Preliminary investigation of platelet activation and turnover in PAPS

3.1 Introduction

Thromboembolism is a common occurrence in patients with the antiphospholipid syndrome. The strong association between APA and thrombosis suggests a direct pathogenic role of these antibodies in promoting thrombosis, and it is probable that several mechanisms are involved as discussed in section 1.3. Platelets are just one of the many potential 'targets' of these antibodies as demonstrated by APA-platelet binding studies (Khamashta *et al*, 1988; Hasselaar *et al*, 1990; Vazquez-Mellado *et al*, 1994), and so it appears reasonable to hypothesise that platelet activation (as a result of such binding) may play a role in the thrombosis of APS. Numerous studies examining platelet activation in this disorder have been published - initial reports measured platelet release products such as βTG or metabolites of thromboxane A_2 and found evidence of *in vivo* platelet activation (Lellouche *et al*, 1991; Wiener *et al*, 1991; Martinuzzo *et al*, 1993; Forastiero *et al*, 1998), however, this has not always been a consistent finding and there are a number of disadvantages when using such methods (section 1.1.4.6). More recently, flow cytometric techniques for detecting platelet activation have been described and platelet activation may be seen in a number of disorders. The expression of degranulation markers such as CD62p and CD63 on the platelet plasma membrane surface can be measured, and some studies have reported significantly higher CD62p values in PAPS patients compared to a group of healthy normal subjects (Fanelli *et al*, 1997).

The purpose of this preliminary investigation was to look for evidence of *in vivo* platelet activation in patients with well documented PAPS using more recently described techniques. As well as using flow cytometric methods (i.e platelet CD62p and CD63 expression), an ELISA technique was used to measure plasma levels of soluble P-selectin, and the DADE PFA-100™ platelet function analyser was employed to detect a possible shortening of closure times (which mimic abnormalities of primary haemostasis). Reticulated platelets were also measured as an indicator of increased platelet turnover and shortened platelet survival.

As mentioned previously, it is unlikely that any one mechanism is solely responsible for the thrombosis of APS. Platelets play a key role in the coagulation system and interact with many cells, including the endothelium. Therefore markers of thrombin generation (i.e. prothrombin fragment F1 +2 and thrombin-antithrombin complexes), and endothelial cell activation (i.e. soluble E-selectin and thrombomodulin), were also measured in an attempt to investigate any possible association with platelet activation. Screening for the inherited thrombophilic genotypes (factor V Leiden and prothrombin gene G20210A variant) were also performed. Many of the patients were receiving aspirin and/or warfarin therapy at the time of the study (which could not be discontinued), hence it was possible to examine the effect (if any) of such therapies on all of these 'activation' markers.

3.2 Methods

3.2.1 Patients

Twenty patients (sixteen female, four male) selected at random, with well documented primary antiphospholipid syndrome (PAPS), regularly attending a routine haemostasis

clinic, were studied. Only patients with stable, 'chronic' disease were included - those suffering from a recent 'acute' event (e.g. thrombosis) were not studied. All patients gave verbal consent to have extra samples of blood collected. Control subjects for the platelet studies were twelve healthy volunteers working in the laboratory, who were not taking any medication at the time of the study.

3.2.2 Blood collection

Patients and controls were rested for approximately 20 minutes prior to venepuncture in order to minimise platelet activation. A Vacutainer® system was used to obtain blood samples, and blood was then collected for platelet activation studies and plasma prepared for specific coagulation tests etc. as outlined in section 2.1.

3.2.3 Flow cytometric studies of platelets

Platelet surface CD42b, CD62p and CD63 expression were measured as described in section 2.2.3, and in this initial study, CD63-PE was used. Reticulated platelets were measured according to the protocol outlined in section 2.2.8. All samples for platelet activation marker studies were analysed within three hours of venepuncture.

3.2.4 Platelet function analysis

Platelet function was assessed using the DADE PFA-100™ platelet function analyser and Collagen/Epinephrine and Collagen/ADP test cartridges (section 2.3).

3.2.5 Enzyme Linked Immunosorbent Assays (ELISA)

All patients had the following parameters measured using an ELISA technique - TAT, F 1+2, soluble P-selectin, soluble E-selectin, soluble thrombomodulin, D-dimer, anticardiolipin antibody and anti-β_2GPI antibody (as described in section 2.4)

3.2.6 Coagulation assays

All patients had the following routine coagulation tests performed on citrated plasma samples - PT, INR, APTT (and mixing study if APTT prolonged), TT and fibrinogen (section 2.6). Lupus anticoagulant testing was performed using a DRVVT and TSVT as described in section 2.7.

3.2.7 Chromogenic factor assays

One patient had chromogenic factor VII and X assays performed on citrated plasma samples using the CA-6000™ Analyser (Sysmex), as described in section 2.8.

3.2.8 Molecular biology tests

All patients had both Factor V Leiden (FVL) and prothrombin gene mutation (PGM) testing performed on DNA samples prepared from buffy coats as described in section 2.9.

3.3 Results

Patient characteristics

The characteristics of the twenty PAPS patients studied are shown in *Table 3.1.* There were sixteen female and four male patients, whose ages ranged from 23 - 75 years. Seventeen (85%) patients had a history of one or more thrombotic events. Of these, 41% were venous thrombosis only, 41% were arterial and 18% were both venous and arterial. Eight of the sixteen women had a history of recurrent miscarriage. Significant thrombocytopaenia (defined as a platelet count $< 100 \times 10^9/l$) had been documented in 15% of patients, either at the time of study or previously. Eighteen patients (90%) were receiving long term aspirin and/or oral anticoagulant therapy.

They had a history of recurrent thromboembolism and/or miscarriage, or were considered to be at a significantly higher risk of thrombotic events. Of these patients, 39% were taking aspirin alone, 22% were taking warfarin, and 39% were receiving both drugs. All patients satisfied the laboratory criteria for APS. The results of APA testing shown in *Table 3.1* reflect the findings on the particular study day - these results will be used in any further statistical analysis, but it is important to note that the LA positivity and ACL titre of a given patient may vary quite significantly over time. Fourteen patients (70%) had a positive LA test. Sixteen patients (80%) had an elevated ACL IgG level, whilst fourteen patients (70%) had an elevated ACL IgM level. Almost half of the patients studied (45%) had antibodies to β_2GPI.

Twelve healthy volunteers were used as control subjects for the platelet studies. There were seven females and five males, and their ages ranged between 21 and 45 years.

Results summary

Median values (with 5th and 95th percentiles) for all assays performed in controls and PAPS patients are listed in *Table 3.2.* Only median values will be referred to in the following text.

Patient	Sex	Age	Thrombosis	Miscarriage	Thrombocytopaenia[a]	Therapy	LA	ACL IgG	ACL IgM	anti-β_2GPI
1	F	23	–	–	+	Aspirin	+	23	87	2.0
2	F	49	Venous	+	+	–	+	59	23	150
3	F	63	Venous and arterial	–	–	Warfarin	+	18	73	2.8
4	F	44	Venous	–	–	Warfarin	+	27	7	1.9
5	F	58	Arterial	+	–	Aspirin and warfarin	–	4	90	9.7
6	F	38	Venous	+	–	Aspirin	+	78	12	51.4
7	F	40	Venous and arterial	+	–	Aspirin and warfarin	+	74	20	32.2
8	F	31	Venous	+	–	Warfarin	+	33	4	29.4
9	F	30	–	+	–	Aspirin	–	13	< 1	2.2
10	M	75	Arterial	–	–	Aspirin and warfarin	+	9	3	2.6
11	F	41	Venous and arterial	–	–	Aspirin and warfarin	–	3	27	2.5
12	M	53	Venous	–	–	Aspirin and warfarin	+	79	58	173
13	F	58	Venous	–	–	Aspirin	+	58	1	18.1
14	M	45	Venous	–	–	–	+	35	39	45.4
15	M	70	Arterial	–	–	Aspirin and warfarin	+	7	< 1	39.7
16	F	38	Arterial	–	–	Aspirin	–	< 1	19	1.9
17	F	40	–	+	+	Aspirin	+	24	83	2.6
18	F	29	Arterial	+	–	Aspirin and warfarin	+	13	< 1	2.8
19	F	27	Arterial	–	–	Warfarin	–	15	< 1	2.3
20	F	47	Arterial	–	–	Aspirin	–	1	15	1.9

Table 3.1 *Clinical and laboratory characteristics of 20 PAPS patients.*

F = female; M = male; + = presence or positive; – = absence or negative; ACL = anticardiolipin level (in either GPL or MPL units) normal range < 5 GPL or < 5 MPL units; LA = lupus anticoagulant; anti-β_2GPI = anti-β_2GPI % concentration (normal range < 2.88%) ; [a] previously documented platelet count of < 100×10^9/l.

Parameter	Controls	PAPS
Platelet count ($\times 10^9$/l)	220 (166, 285)	227 (127, 345)
CD62p (%)	4.0 (1.3, 8.0)	4.5 (1.8, 17.2)
CD63 (%)	10.1 (7.5, 13.9)	14.3 (9.9, 18.9)
Reticulated platelets (%)	10.3 (7.5, 11.2)	10.9 (6.6, 15.1)
CT Coll/ADP (sec)	84.5 (66.9, 100.1)	78.5 (64.5, 116.3)
CT Coll/EPI (sec)	107.0 (89.5, 133.2)	149.0 (81.8, 248.0)
soluble P-selectin (ng/ml)	18.8 (8.5, 36.8)	34.0 (16.8, 78.7)
TAT (μg/ml)	2.4 (1.1, 4.8)	2.7 (1.1, 12.2)
F 1+2 (nmol/l)	0.8 (0.4, 1.6)	0.4 (0.1, 1.4)
D-dimers (ng/ml)	0.6 (0.0, 25.3)	9.6 (0.0, 221.9)
soluble E-selectin (ng/ml)	32.8 (20.4, 57.4)	42.4 (18.4, 86.5)
Thrombomodulin (ng/ml)	50.9 (27.2, 69.1)	66.4 (40.8, 162.1)

Table 3.2 *Median values (with 5^{th} and 95^{th} percentiles) for all assays performed in controls and PAPS patients*

Expression of platelet activation markers

The gating of platelets was highly satisfactory for both patients and controls - the median values for CD42b expression were 98.9% for PAPS patients and 99.0% for controls. Results for the percentage of platelets expressing CD62p and CD63 are shown in *Figures 3.1 and 3.2* respectively. The median values for CD62p expression were similar in both groups, with no sigificant difference - 4.0% for the control group, and 4.5% for the PAPS patient group. Notably, two of the PAPS patients had relatively high levels of CD62p expression (16.9% and 21.6%).

There was a significant difference in median values for CD63 expression which were 10.1% for controls and 14.3% for patients (p=0.0008). At the time of the study, three patients had a platelet count < 150×10^9/l. Median values for their CD62p and CD63 expression were 4.2% and 18.8%, respectively, in comparison to the corresponding values of 4.6% and 14.1% in patients with a normal platelet count.

In the patient group, there was no correlation between the values obtained for CD62p and CD63 expression.

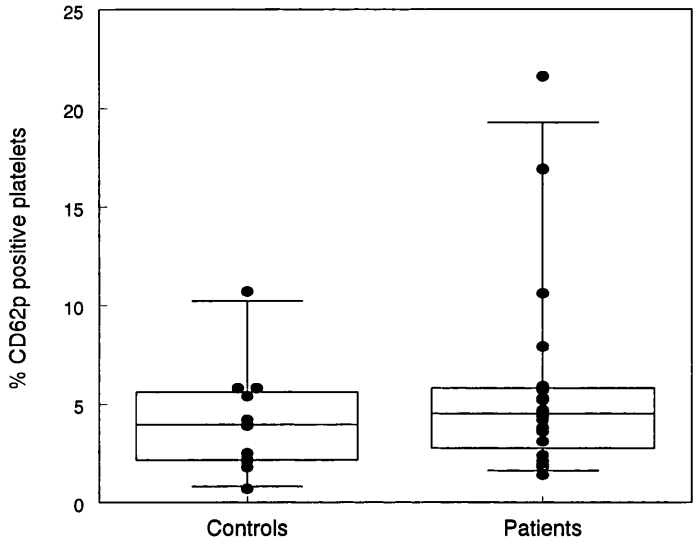

Figure 3.1 *% platelets expressing CD62p in controls (n=12) and patients (n=20).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p = ns

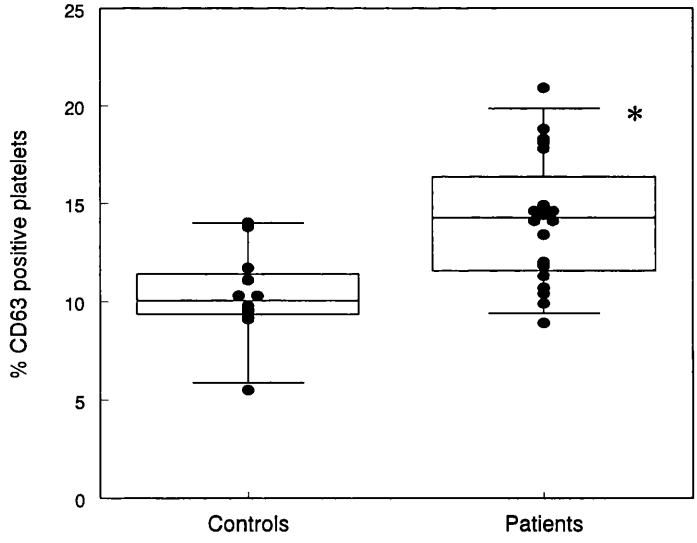

Figure 3.2 *% platelets expressing CD63 in controls (n=12) and patients (n=20).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.0008

Patients receiving aspirin therapy

In the PAPS group, 14 patients were receiving chronic daily aspirin therapy at the time of the study. *Figures 3.3 and 3.4* demonstrate the results of CD62p and CD63 expression in patients receiving aspirin in comparison to patients not on aspirin, and controls. Median values for CD62p expression were similar amongst the three groups - values were 4.0% in controls, 4.7% in PAPS patients on aspirin, and 4.0% in PAPS patients not on aspirin. Interestingly, the two patients with the highest levels of CD62p expression were not receiving aspirin.

The median values for CD63 expression were 10.1% in controls, 13.1% in PAPS patients on aspirin, and 18.0% in PAPS patients not on aspirin. The difference in median CD63 expression between PAPS patients on aspirin and those who were not, reached statistical significance (p=0.02), however it is important to note that the groups are small and mismatched, and so there may be a selection bias. Notably, the patient with the highest level of CD63 expression (20.9%) was taking aspirin therapy at the time of study.

Relationship of platelet activation with APA 'markers' and thrombosis

There was no correlation between either CD62p or CD63 expression and the presence or absence of ACL antibodies, LA activity or antibodies to β_2GPI. In those patients with ACL and anti-β_2GPI antibodies, there was no correlation between the level of CD62p or CD63 expression and the antibody titre. There was no significant difference in platelet CD62p or CD63 expression between patients with a history of arterial thrombosis and those without. One interesting observation was that nine PAPS patients had antibodies to β_2GPI, and of these, seven (78%) had a history of venous

thrombosis. This was in contrast to eleven patients without anti-β_2GPI antibodies, of whom only three (27%) had suffered venous thrombosis previously.

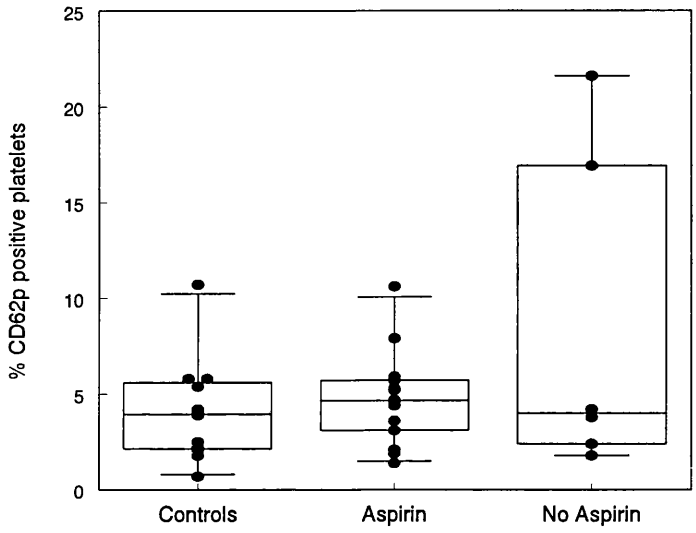

Figure 3.3 *% platelets expressing CD62p in controls (n=12) and in patients either receiving aspirin (n=14) or no aspirin (n=6).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p = ns (all groups)

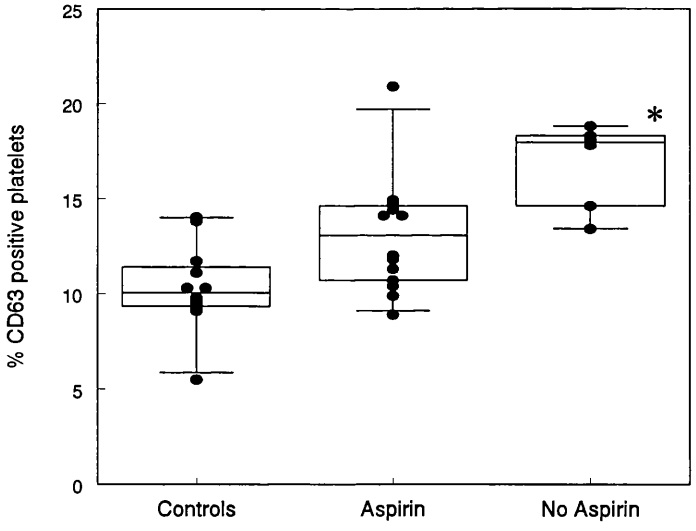

Figure 3.4 *% platelets expressing CD63 in controls (n=12) and in patients either receiving aspirin (n=14) or no aspirin (n=6).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.02 (Asp *vs* No Asp)

Plasma soluble P-selectin

Plasma soluble P-selectin levels were significantly higher in PAPS patients compared to controls (p=0.003) as seen in *Figure 3.5.* The median levels were 18.8 ng/ml in controls and 34.0 ng/ml in PAPS patients. There was no significant difference in median levels of soluble P-selectin between PAPS patients on aspirin and those not on aspirin. There was no correlation between soluble P-selectin levels and either platelet CD62p or CD63 expression in PAPS patients.

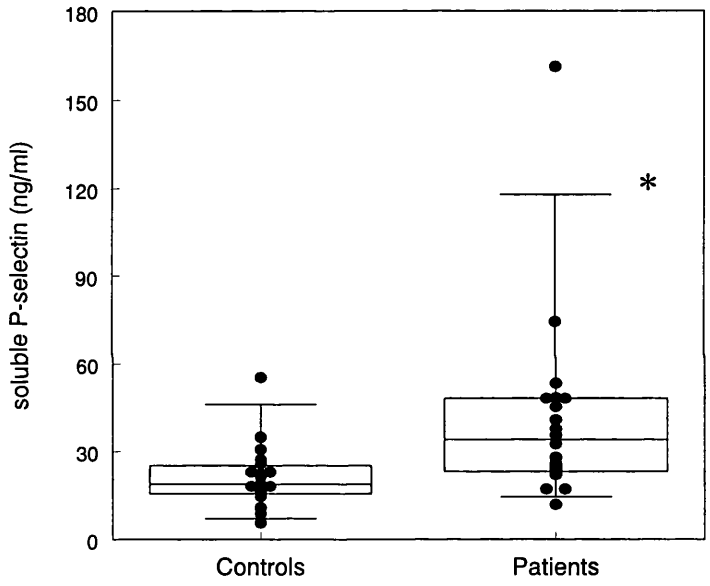

Figure 3.5 *Plasma soluble P-selectin levels in controls (n=20) and patients (n=20).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.003

Reticulated platelets

There was no significant difference in reticulated platelet percentages between controls and PAPS patients as illustrated in *Figure 3.6.* Median values were 10.3% in controls, and 10.9% in patients. There was no correlation between reticulated platelet percentages and levels of either CD62p or CD63 expression. The one patient who had

significant thrombocytopaenia at the time of the study (platelet count 50 × 10^9/l) had a normal reticulated platelet count of 11.0%.

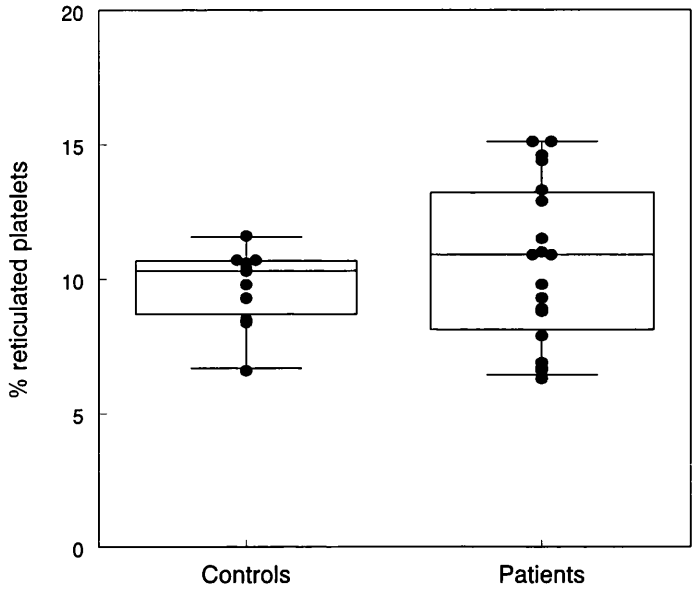

Figure 3.6 *% reticulated platelets in controls (n=11) and patients (n=19).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p = ns

PFA-100™ platelet function analysis

Results of PFA-100™ platelet function analyser closure times using both Collagen/ADP and Collagen/Epinephrine cartridges in controls and PAPS patients are illustrated in *Figures 3.7 and 3.8.* In general, there was no significant shortening of closure times noted. Median values for Coll/ADP were 84.5s in controls, and 78.5s in PAPS patients (p=ns). One patient had a Coll/ADP CT of 56s, which was below the normal range. Levels of platelet CD62p and CD63 expression in this patient were 1.8% and 13.4% respectively, and interestingly this patient had the highest level of soluble P-selectin (161.3 ng/ml). The difference in closure times for Coll/Epi between controls (median 107s) and PAPS patients (median 149s) was significant (p=0.0003),

and this was probably due to the predictable effect of aspirin on Coll/Epi cartridge as seen in *Figure 3.9.*

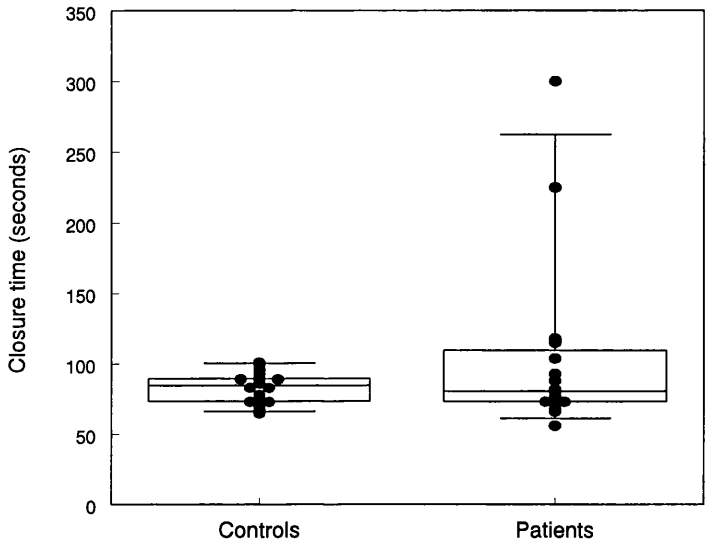

Figure 3.7 *Closure time for Coll/ADP in controls (n=20) and patients (n=20).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p = ns

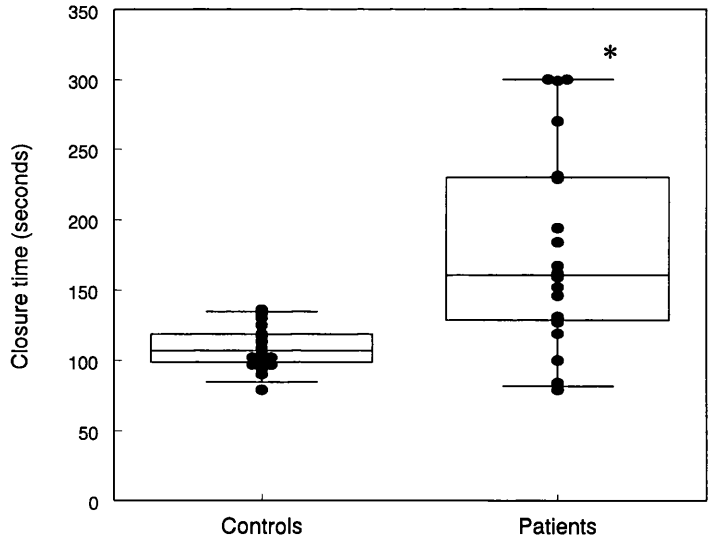

Figure 3.8 *Closure time for Coll/EPI in controls (n=20) and patients (n=20).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.0003

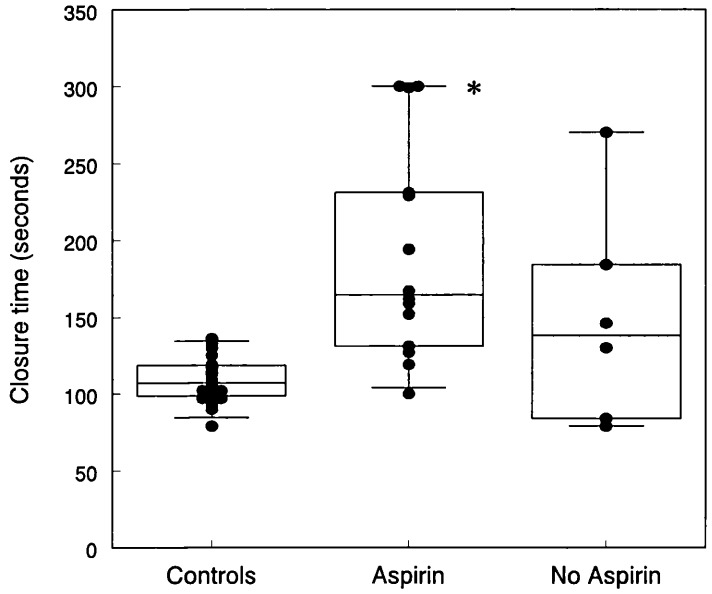

Figure 3.9 *Closure time for Coll/EPI in controls (n=20) and in patients either receiving aspirin (n=14) or no aspirin (n=6).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p <0.0001 (Asp *vs* Cont)

Thrombin generation markers and D-dimer formation

Results for the levels of TAT, F 1+2 and D-dimers are shown in *Figures 3.10, 3.11 and 3.12.* The median values for TAT were 2.35 μg/ml in controls, and 2.70 μg/ml in the PAPS patients group (p=ns). F 1+2 levels were significantly lower in PAPS - median values were 0.43 nmol/l in patients, and 0.80 nmol/l in controls (p=0.01). Median D-dimer levels were 0.60 ng/ml in controls, and 9.6 ng/ml in PAPS patients (p=ns). Seven patients had TAT levels above the normal range, and of these patients, four also had elevated levels of F 1+2. D-dimer levels were significantly elevated in two patients, both of whom had elevated levels of TAT.

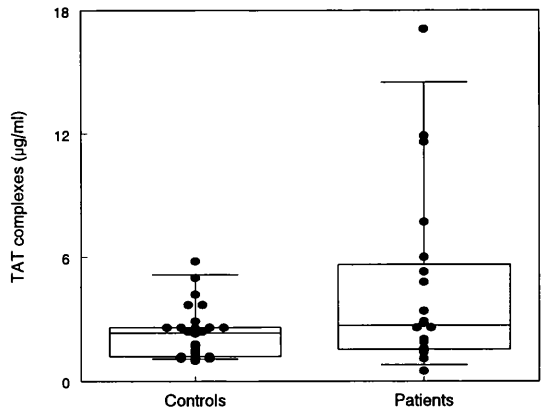

Figure 3.10 *Plasma levels of TAT complexes in controls (n=26) and patients (n=20).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p = ns

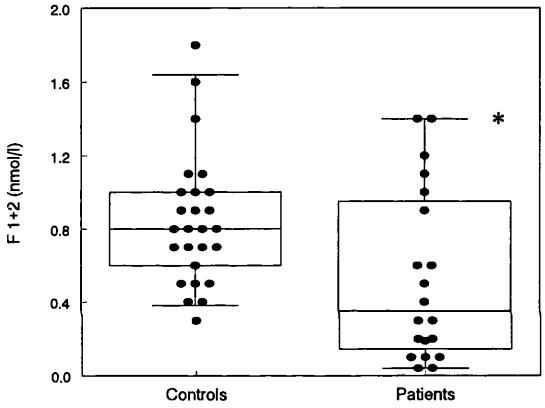

Figure 3.11 *Plasma levels of F 1 + 2 in controls (n=26) and patients (n=20).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.01

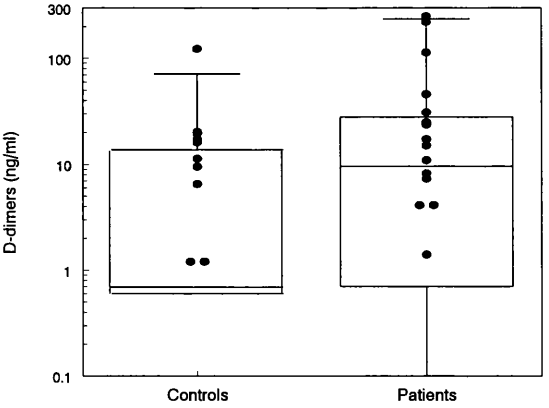

Figure 3.12 *Plasma D-dimer levels in controls (n=20) and patients (n=20).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p = ns

There was a significant positive correlation between TAT and F 1+2 levels (Spearman Rank Correlation Coefficient [rs]=0.72, p=0.0003), and TAT and D-dimer levels (rs=0.49, p=0.03) in PAPS patients as shown in *Figures 3.13 and 3.14.*

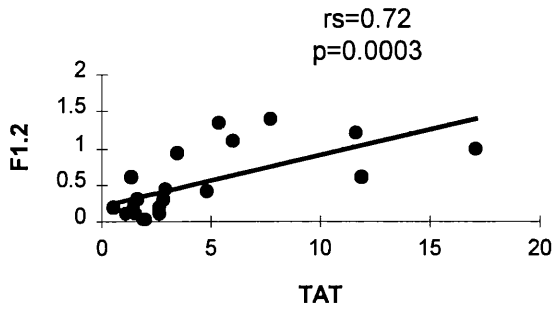

Figure 3.13. *Correlation between TAT and F 1+ 2 levels in PAPS patients*

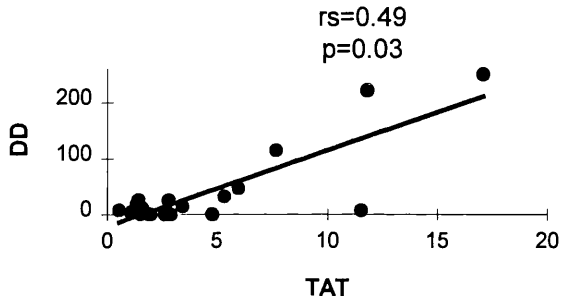

Figure 3.14 *Correlation between TAT and D-dimer levels in PAPS patients*

Patients receiving oral anticoagulant therapy

In the PAPS group, 11 patients were receiving long term oral anticoagulant therapy with warfarin at the time of the study. *Figures 3.15 and 3.16* demonstrate the results of TAT and F 1+2 levels in patients on warfarin in comparison to patients not on warfarin, and controls. Median values for TAT were 2.35 μg/ml in controls, 1.9 μg/ml in patients on warfarin, and 5.3 μg/ml in patients not on warfarin. The difference in TAT levels between warfarinised and non-warfarinised patients was significant (p=0.007). Median values for F 1+2 were 0.80 nmol/l in controls, 0.20 nmol/l in patients on warfarin, and 1.0 nmol/l in patients not on warfarin. The difference in

F 1+2 levels between warfarinised and non-warfarinised patients was significant (p=0.004). There was no significant difference in D-dimer levels between warfarinised and non-warfarinised patients (results not shown).

There were no significant differences in median levels of platelet activation markers (i.e. CD62p, CD63 and sP-selectin) between warfarinised and non-warfarinised patients (results not shown).

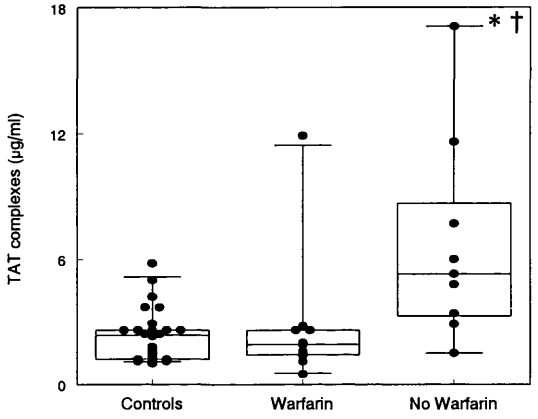

Figure 3.15 *Plasma levels of TAT complexes in controls (n=26) and in patients either receiving warfarin (n=11) or no warfarin (n=9).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.007 (No Warf *vs* Warf) and † p = 0.001 (No Warf *vs* Cont)

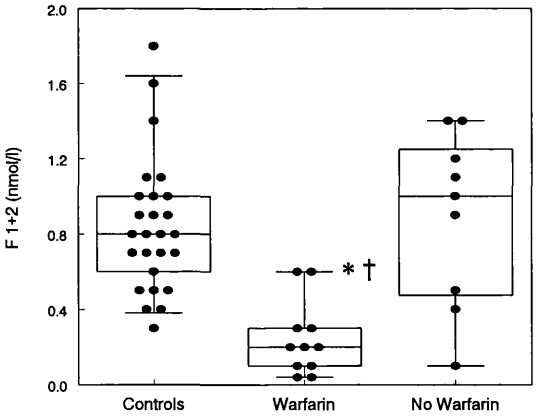

Figure 3.16 *Plasma levels of prothrombin fragment 1 + 2 in controls (n=26) and in patients either receiving warfarin (n=11) or no warfarin (n=9).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.004 (Warf *vs* No Warf) and † p < 0.0001 (Warf *vs* Cont)

Correlation between INR and TAT and F 1+2 levels

There was a hyperbolic relationship between INR and either TAT or F 1+2 levels, as seen in *Figure 3.17.* This was highly significant for INR vs F 1+2 (p=0.009), and just failed to reach statistical significance for INR vs TAT (p=0.05). There was no significant correlation between INR and the level of D-dimer. Notably, one patient on warfarin had an elevated TAT level of 11.9 μg/ml despite having a 'therapeutic' INR of 3.84 at the time of sampling. Repeat INR testing using other thromboplastin reagents (Thromboplastin IS and Recombiplastin) gave comparable INR results of 3.4 and 3.51 respectively. Factor VII and X assays were subsequently performed on this patient using chromogenic assays, and levels of 25.3 U/dl (factor VII) and 17.3 U/dl (factor X) were obtained. These levels were consistent with the 'degree' of anticoagulation, and varying dilutions of the plasma sample did not indicate the presence of an inhibitor.

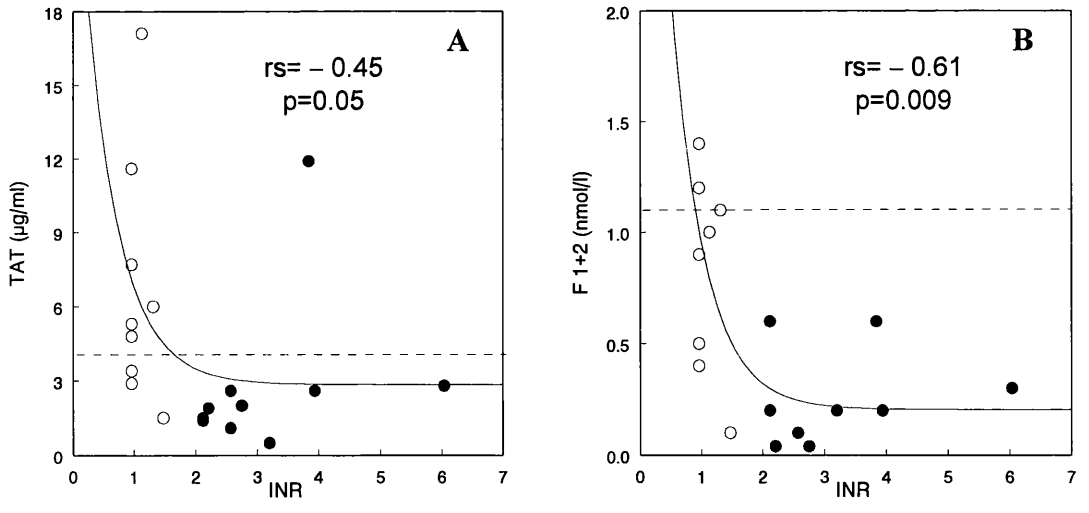

Figure 3.17 A and B *Correlation between INR and TAT, and INR and F 1+2 levels in PAPS patients.*
Dotted horizontal line indicates upper limit of kit assay normal range. Open circles indicate non-warfarinised patients; closed circles indicate warfarinised patients.

Endothelial cell 'activation' markers

Results of soluble E-selectin and thrombomodulin levels in controls and patients are shown in *Figures 3.18 and 3.19.* The median values for soluble E-selectin were 32.8 ng/ml in controls, and 42.4 ng/ml in PAPS patients, and this difference failed to reach statistical significance (p=0.06). Thrombomodulin levels were significantly higher in patients compared to controls (p=0.0001); median values were 66.4 ng/ml in patients, and 50.9 ng/ml in controls. Since TM levels are known to be increased in patients with renal impairment, results were interpreted with this in mind. Levels of TM and serum creatinine (routinely performed on 15 patients only) are listed in *Table 3.3.* The normal range for creatinine is 62-133 μmol/l, and three patients had creatinine levels above the normal range. There was a significantly positive correlation between thrombomodulin levels and serum creatinine for all patients (rs = 0.79, p = 0.0004), however no correlation was found between serum creatinine and levels of E-selectin.

Thrombomodulin (ng/ml)	Serum creatinine (μmol/l)
77.2	76
59.9	71
55.6	85
83.1	112
160.5	136
69.4	97
63.1	76
59.6	65
102.6	125
72.1	119
66.5	75
193	177
93.7	143
56.2	106
118.6	134

Table 3.3 *Thrombomodulin and creatinine levels in 15 PAPS patients*

There was no correlation between levels of soluble E-selectin and thrombomodulin. For both of these parameters, there was no significant difference in median levels between patients on aspirin or no aspirin, and patients on warfarin or no warfarin.

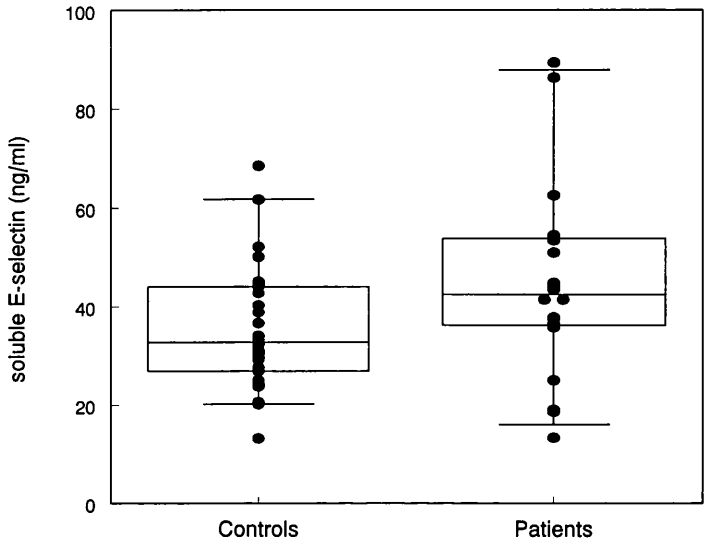

Figure 3.18 *Plasma soluble E-selectin levels in controls (n=30) and patients (n=20).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p = ns

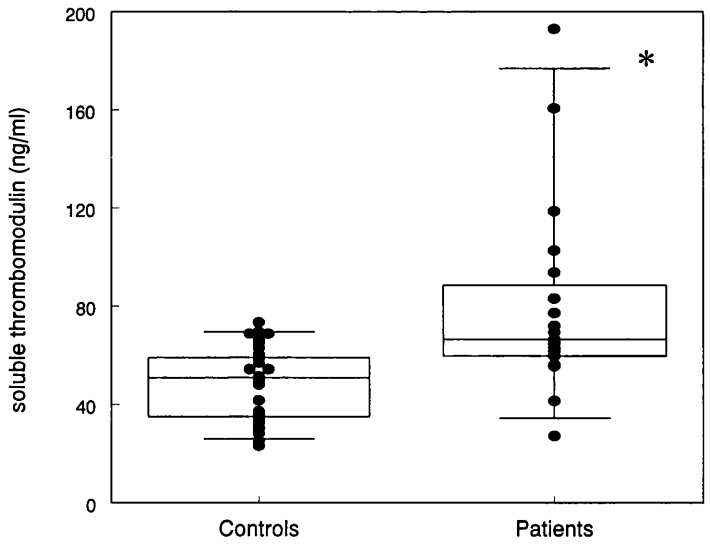

Figure 3.19 *Plasma thrombomodulin levels in controls (n=34) and patients (n=20).* Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.0001

Plasma fibrinogen levels

The median values for fibrinogen were 2.76 g/l in patients, which were not elevated and fell within the normal range in our laboratory (1.5 - 4.5 g/l). There was no signficant difference in fibrinogen levels between warfarinised and non warfarinised patients, and there was no correlation with the thrombin generation markers TAT and F1 + 2.

Correlation between platelet activation, thrombin generation and endothelial activation markers

All parameters (i.e. platelet activation markers, thrombin generation and endothelial activation markers) were examined to assess if there was any correlation between them. Apart from those mentioned previously, the only significant correlation was a negative association between platelet CD63 expression and plasma F 1+2 levels *(Figure 3.20).*

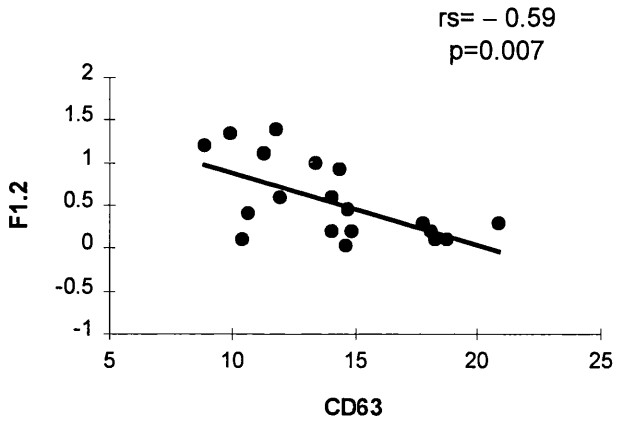

Figure 3.20 *Correlation between platelet CD63 and F 1+2 levels in PAPS patients*

FVL and PGM results

All patients had FVL and PGM testing performed. One patient was found to be heterozygote for the FVL mutation, whilst none of the twenty patients studied had the PGM detected. The patient with FVL had a history of arterial thrombosis only, and did not have a more severe form of thrombotic disease compared with the other patients.

3.4 Discussion

The mechanisms responsible for thrombosis in patients with APS remain unclear, and it is unlikely that any one, single mechanism is responsible for the thrombogenic activity of all APA's. Many 'haemostatic' parameters which utilise phospholipids for their coagulant/anticoagulant activity have been assayed in patients with APA, and in many cases, significant abnormalities have been reported. One theory proposes that activated platelets may play an important role in thrombus formation in this condition. There have been several previous studies which have measured the expression of platelet activation markers in APS using flow cytometric techniques. One group examined both CD62p and CD63 expression in the platelet rich plasma of 16 PAPS patients, and found a statistically significant increase in the expression of CD62p above the normal cut off in 56% patients (Fanelli *et al*, 1997). No difference was found for CD63 expression between normal subjects and PAPS patients. Another study also failed to show any increase in lysosomal membrane exposure (i.e.CD63 expression) in the platelet rich plasma of seven APA positive patients with thromobcytopaenia (Out *et al*, 1991), however CD62p was not measured.

In this preliminary investigation, the expression of platelet CD62p and CD63 was measured in unmanipulated, whole blood samples taken from 20 patients with

well documented PAPS. There was no significant difference in the median value for CD62p expression in controls and patients, however median CD63 expression was significantly higher in patients compared to controls. This was a somewhat unexpected finding, since it is known that higher concentrations of agonists (such as thrombin) are required for dense granule or lysosomal enzyme secretion than α-granule secretion (Kaplan 1994), and so one would expect to find elevated levels of platelet CD62p as well. There are however, several possibilities which may explain these findings. A recent study has reported that circulating degranulated platelets rapidly lose their surface P-selectin to the plasma pool, but continue to circulate and function (Michelson *et al*, 1996). Thus it is quite feasible that platelets may continually circulate in a 'heightened' state of activation, but express normal levels of CD62p. Another possibility is that the platelets which may be expressing higher levels of CD62p, bind preferentially to leukocytes (monocytes and neutrophils) (McEver 1991), and so would have been excluded from the flow cytometric analysis. In this investigation, plasma levels of soluble P-selectin were significantly higher in PAPS patients compared to controls, however, there was no correlation with platelet CD63 expression. Reasons for this apparent lack of association between the two parameters, include the possibility that antiplatelet drugs (i.e. aspirin) may have variable effects on the expression of different activation markers (discussed later), and that activated endothelial cells may also be a source of soluble P-selectin (Fijnheer *et al*, 1997).

Although the findings in this study are in contrast to those from the studies cited previously, it may be inappropriate to compare the results, since a whole blood method was used in this current investigation. Indeed, a recently published paper

which used a whole blood flow cytometric method, also found no significant increase in P-selectin on the outer surface of circulating platelets in APS patients (Shechter *et al*, 1998). However, the investigators did report a significantly lower concentration of serotonin in the platelets of APS patients compared to controls, suggesting that platelets in APS patients circulate in an activated state. In the other studies which used platelet rich plasma, centrifugation and washing steps were performed, and such manipulation of samples may affect the expression of platelet activation markers, and also result in the selective loss of larger platelets, platelet-platelet aggregates, and platelet-leukocyte aggregates.

Patients receiving aspirin therapy had significantly lower median values for CD63 expression than those who were not on aspirin, although patient numbers were small. Although median CD62p values were similar for both patient groups, the two patients with the highest CD62p levels were not receiving aspirin. An interesting finding was that the patient with the highest level of CD63 expression (20.9%), was on aspirin therapy at the time of the study. Explanations for this result could be that the patient was on too low a dose of aspirin or even non-compliant with therapy; however it is possible that despite 'adequate' aspirin dosage, the patients platelets were still able to be activated. This is not surprising, given the fact that platelet activation is a complex process and aspirin inhibits only one modulatory pathway effecting platelet activation. Indeed, one study has reported that in a group of controls, the expression of platelet CD62p, CD63 and fibrinogen binding in response to agonists such as ADP and thrombin, does not alter with aspirin therapy (Chronos *et al*, 1994). Hence aspirin therapy is probably only partially effective in preventing platelet activation in some PAPS patients.

Antibodies to β_2GPI have been shown to be significantly associated with a history of thrombosis in APS patients (McNally *et al*, 1995a; Martinuzzo *et al*, 1995; Tsutsumi *et al*, 1996; Forastiero *et al*, 1997), and this was also the case in the current group of 20 PAPS patients. However, in this investigation there was no significant difference in either platelet CD62p or CD63 expression between those patients who had antibodies to β_2GPI, and those who did not.

No significant difference in reticulated platelet percentages was found between the patient group and controls, and there was no correlation between numbers of reticulated platelets and levels of either CD62p or CD63 expression. Thus there was no evidence to support an increase in platelet turnover (and thus platelet consumption), even in the presence of low levels of circulating activated platelets.

Platelet function as analysed on the PFA-100™, did not reveal a significant reduction in closure times. In most cases, patients on aspirin therapy had prolonged closure times when tested with the Collagen/Epinephrine cartridge. Although one patient had a slightly shortened closure time on Coll/ADP testing, closure times were probably not generally reduced in PAPS patients, since the slight (but significant) increase in platelet activation (as measured by CD63 expression) was unlikely to affect this assay.

As well as examining platetet activation in these PAPS patients, the degree of activity of the procoagulant system was also measured by assaying plasma levels of TAT, F1 +2, and D-dimers. Numerous studies have documented variable degrees of increased thrombin generation in patients with APS. One cross-sectional study of 18 thrombotic patients with PAPS and 18 nonthrombotic subjects with APA, found increased F1+2 and D-dimer levels in both patient groups compared to controls,

whereas TAT levels did not differ (Ames *et al*, 1996). The patients had had their oral anticoagulants suspended for the study. One reason cited for the discrepancy between TAT and F1+2 levels, was that there may be a defective function of ATIII-heparan sulphate in patients with APA, in keeping with the notion that binding of APA to heparan sulphate can prevent formation of TAT complexes (Shibata *et al*, 1994) and in agreement with a recent report showing higher levels of TAT in SLE patients without APA compared to those with APA (Yamazaki *et al*, 1994). In contrast, another study found significantly increased levels of TAT and only slightly increased levels of F1+2 in LA positive patients (Falanga *et al*, 1994), none of whom were receiving oral anticoagulants at the time. In this investigation of 20 PAPS patients, none of the subjects receiving warfarin therapy had it suspended for the study, hence the results should be interpreted with this in mind. Median TAT levels were significantly higher in non-warfarinised patients compared to controls, and also compared to patients on warfarin. On the other hand, median F 1+2 levels were significantly reduced in warfarinised patients compared to controls and those not on warfarin, however, there was no significant difference in median F 1+2 levels between non warfarinised patients and controls. In other words, when compared to control subjects, warfarin appeared to have a greater effect on reducing patients F 1+2 levels than it did on their TAT levels. The reasons for this are unclear - it may be that warfarin affects F1+2 production to a greater extent than it does TAT formation, and it is also possible that there may be more than one form of TAT complex in normal human plasmas, and that ELISA kits may not necessarily detect the dominant form. Nevertheless, there was a significant positive correlation between TAT and F 1+2 levels in the patients. Although median levels of D-dimer were not significantly

different in patients compared to controls, there were several patients with elevated levels of D-dimers, and levels of D-dimers were also positively correlated with both TAT and F 1+2. It may be that D-dimers are a less sensitive indicator of thrombin generation than either TAT or F 1+2.

There was a negative correlation (which apperared to be exponential) between a patients INR and both TAT and F 1+2 levels, although it reached statistical significance for INR *vs* F 1+2 only. Numerous studies have investigated the relationship between INR level and markers of thrombin generation, and some have proposed that they may be used to monitor efficacy of oral anticoagulant therapy. One study which measured TAT and F1+2 in anticoagulated patients, found significantly higher values in controls than in patients, but there were no significant differences in either of these marker values among patients with different INR's, nor in the same patients studied several times (Barcellona *et al*, 1997). This is in direct contrast to another study of 79 oral anticoagulant patients, whereby the mean values of F 1+2 decreased in parallel with the intensity of warfarin therapy at four different INR intervals (<2, 2-3, 3.1-4, >4) (Mannucci *et al*, 1991). In the current investigation, one patient who appeared to be adequately warfarinised (INR 3.84), had an elevated TAT level of 11.9 μg/ml. Some authors have suggested that the prothrombin time test may be affected by the presence of a lupus anticoagulant (Moll & Ortel 1996; Della Valle *et al*, 1996), however other investigators have found that if sensitive thromboplastin reagents are used together with instrument-specific ISI values, there is no apparent effect of lupus anticoagulants on the INR (Lawrie *et al*, 1997). A recent study has demonstrated similar results, although it reported some inaccuracies of INR determination with the Innovin reagent (Robert *et al*, 1998). In order to further assess

the intensity of anticoagulation in the patient mentioned above, INR testing was performed with two other thromboplastins, and chromogenic factor VII and X assays were also performed. These confirmed that the patient was indeed adequately anticoagulated, hence it would appear that in some patients, significant thrombin generation may still occur despite warfarin therapy. The only other possible explanation is that in some circumstances, proteins induced by vitamin K antagonists (PIVKA's) may interfere with the ELISA determination of TAT/F1 + 2 complexes.

There were no significant differences in levels of platelet activation markers (i.e. CD62p, CD63 and sP-selectin) between warfarinised and non-warfarinised patients. In those patients not receiving warfarin, levels of thrombin generation markers did not correlate with levels of platelet activation markers, hence there was no evidence to support the possibility that thrombin may mediate the platelet activation found in APS.

Several groups have measured fibrinogen levels (using the Clauss method) in patients with APA, and have reported varying results. One study reported that patients with LA and a history of thrombosis had significantly higher fibrinogen levels than LA patients without thrombosis (Gschwandtner *et al*, 1996). They postulated that the increased fibrinogen levels were not related to disease activity in patients with autoimmunity, because only two of eight patients with high fibrinogen levels had an autoimmune disease. Another study which examined 144 patients with APA, found that plasma levels of fibrinogen (and vWF) were associated with the occurrence of arterial and venous thrombosis (Ames *et al*, 1995). Plasma levels of fibrinogen in thrombotic APA patients were found to be significantly higher than those of a control group of thrombotic patients who suffered thrombosis for other reasons, indicating

that APA may account for the difference. It has also been suggested that elevated levels of fibrinogen may be regarded as a marker of increased thrombin formation (Ceriello *et al*, 1994). In the current study, there was no evidence of elevated fibrinogen levels in PAPS patients, and no correlation with TAT and F 1+2. Since only three of the twenty patients had no history of thrombosis, it was not possible to determine whether or not there were increased fibrinogen levels in the thrombotic subgroup of patients in comparison to the non thrombotic group.

Both increased levels of plasma E-selectin and thrombomodulin (TM) reflect endothelial cell injury and/or activation, and in renal failure, TM levels are significantly correlated with levels of creatinine (Boffa & Karmochkine 1998). In the current study, median values of plasma TM were signficantly higher in PAPS patients compared to contols, and although none of the PAPS patients had severe renal impairment, there was a significant correlation between TM levels and serum creatinine for all patients. Although the difference in E-selectin levels betwen patients and controls failed to reach statistical significance, there were some individual patients with high E-selectin values. Interestingly, there was no correlation between E-selectin and TM levels in PAPS patients. This may be partly due to the effect of renal dysfunction on TM levels, or differences in half-lives, but it may also be because the release of these two soluble markers from the endothelium is under the control of different factors, and so they may indeed reflect different aspects of endothelial cell injury.

An analysis of all assayed parameters demonstrated a significantly negative correlation between platelet CD63 expression and plasma F 1+2 levels. It is difficult to explain this finding - one possibility is that patients receiving aspirin therapy (who

had signficantly lower CD63 values) may be less likely to be receiving concomitant warfarin therapy, thus accounting for an increase in plasma F 1+2 levels; however, 39% patients were receiving both medications, hence this is an unlikely explanation. It is possible that there may be correlations between other variables which are inapparent or 'hidden' due to the effects of both aspirin and warfarin on the parameters measured, however it was not possible to discontinue either of these medications for the purposes of this study.

Screening for the FVL mutation and the prothrombin gene G20210A mutation (PGM) detected one PAPS patient who was heterozygous for FVL mutation only. This patient had a history of arterial thrombosis (which was not particularly severe), and was receiving aspirin therapy. Case reports of individuals and families who have coexistence of both APS and FVL have been published, and in one case report, the patient was found to have severe arterial thrombotic disease (Picillo *et al*, 1998). Studies of larger groups of APS patients have reported variable findings. One group found that the prevalence of FVL was higher in APA thrombotics (14%) and non-APA thrombotics (18%) compared to controls (4%) (Ames *et al*, 1998). Another study which analysed 60 patients with APA for the presence of FVL, found four heterozygotes and one homozygote amongst 26 patients with a history of venous thrombosis, and none in 34 patients without venous thrombosis (13 of whom had arterial thrombosis) (Montaruli *et al*, 1996). Thus the incidence of FVL was signficantly elevated in patients with APA and venous thrombosis, suggesting that FVL may play an important role in the occurrence of venous thrombosis in patients with APA. On the other hand, several other studies have failed to demonstrate a significantly increased prevalence of FVL amongst PAPS patients with thrombosis

(Dizon-Townson *et al*, 1995; Pablos *et al*, 1999). Differences in patient selection probably accounts for these discrepancies, however it is apparent that the presence of FVL or PGM are not prerequisites for the thrombotic events in patients with APS, since thrombosis occurs in patients without these mutations.

In conclusion, the observations made in this preliminary chapter suggest that there is *in vivo* platelet activation occurring in some patients with PAPS, as evidenced by platelet CD63 expression and plasma levels of soluble P-selectin. Although aspirin treated patients (as a group) have significantly lower levels of CD63 expression than those not on aspirin, it is apparent that platelet activation can occur despite the use of such therapy. There is also evidence of increased thrombin generation in PAPS, and treatment with warfarin appears to be effective in reducing levels of TAT and F 1+2 in most cases. Endothelial cell activation (as demonstrated by increased TM levels) is apparent in this group of patients, and may partly contribute to the elevated levels of soluble P-selectin. As mentioned previously, platelet activation is a complex process, and a number of novel flow cytometric methods for determining such activation have recently been published, and were adapted for use in the laboratory. Therefore in the next chapter, these methods were used to look for further evidence of platelet activation in APS. In order to determine whether or not some of the observed changes noted in this chapter may be due to autoimmunity, a group of patients with SLE (and a smaller group with rheumatoid arthritis) were also investigated. The results of these experiments are presented in chapter 4.

4. Further analysis of platelet activation and haemostatic changes in PAPS and SLE

4.1 Introduction

In the previous chapter, evidence of increased platelet and endothelial cell activation was found in a significant number of patients with PAPS. As this study was only a preliminary investigation, the initial assessment of platelet activation had been made by measuring expression of the two degranulation markers CD62p and CD63, and plasma levels of soluble P-selectin. However, platelet activation involves many other events (e.g. aminophospholipid exposure and microparticle formation), hence it is possible that measuring degranulation markers alone may limit the ability to detect platelet activation under all circumstances. Whilst performing the initial work for this thesis, a number of methods were published which allow the detection of different aspects of platelet activation (Combes *et al*, 1997; Metcalfe *et al*, 1997; Ruf *et al*, 1997; Furman *et al*, 1998). They include the measurement of annexin V binding to platelets (which detects phosphatidylserine exposure on the outer leaflet of the platelet plasma membrane); PAC-1 binding to platelets (which detects a conformational change in the GP IIb/IIIa complex upon activation); as well as measuring numbers of circulating platelet-leukocyte complexes and platelet microparticles. Some of these methods were adapted for use in the laboratory and their development is outlined in detail in Chapter 2. Several of the assays have been used to detect platelet activation in other clinical conditions including coronary artery disease (Furman *et al*, 1998), haemodialysis (Gawaz *et al*, 1994), and heparin induced thrombocytopaenia (Lee *et*

al, 1996; Tomer 1997). Since the preliminary results described in Chapter 3 demonstrated some evidence of *in vivo* platelet activation in PAPS, it was considered that a more extensive investigation of platelets using these flow cytometric methods may reveal further evidence of heightened platelet activity in PAPS, as well as providing some information about the possible nature of platelet activation in this disorder.

As mentioned previously, APS may occur in association with other autoimmune diseases. In particular, SLE is the condition most frequently associated with secondary APS. Thrombosis in SLE patients with APA is a common finding, however it has been reported that SLE patients without APA are also at an increased risk of thrombosis (McNeil *et al*, 1991). Hence in this chapter, a group of patients with longstanding SLE (both APA positive and APA negative) were also examined in order to detect any abnormalities in platelet activation, as well as to provide a 'control' autoimmune group for comparison with PAPS patients. In one assay (i.e. detection of platelet-leukocyte complexes), patients with another longstanding autoimmune disorder (rheumatoid arthritis) were also studied.

As well as measuring platelet activation, thrombin generation was also measured in SLE patients as a number of haemostatic changes have been previously described in this disorder (Keeling & Isenberg 1993). Many of the SLE patients were receiving immunosuppressive therapy, hence an effect (if any) of this therapy on 'activation' markers could not be excluded.

4.2 Methods

4.2.1 Patients

Twenty patients with PAPS, thirty patients with SLE, and ten patients with rheumatoid arthritis (RA) regularly attending routine haemostasis or rheumatology clinics were studied. Only patients with stable, 'chronic' disease were included - those suffering from a recent 'acute' event (e.g. thrombosis) were not studied. All patients gave verbal consent to have extra samples of blood collected. Control subjects for the platelet studies were twenty healthy volunteers working in the laboratory, who were not taking any medication at the time of the study.

4.2.2 Blood collection

Patients and controls were rested approximately for 20 minutes prior to venepuncture in order to minimise platelet activation. A Vacutainer® system was used to obtain blood samples, and blood was then collected for platelet activation studies and plasma prepared for specific coagulation tests etc. as outlined in section 2.1.

4.2.3 Flow cytometric studies of platelet activation

Platelet surface CD42b, CD62p and CD63 expression were measured as described in section 2.2.3, and in this study, CD63-FITC was used. PAC-1 and Annexin V binding to platelets were measured as described in sections 2.2.4 and 2.2.5. Platelet leukocyte complexes were performed as described in section 2.2.6 and platelet derived microparticles were measured in plasma samples according to the method outlined in section 2.2.7. All samples for platelet activation studies were analysed within 3 hours

of venepuncture. All of the above tests were performed on PAPS and SLE patients, whilst RA patients only had levels of platelet-leukocyte complexes determined.

4.2.4 Enzyme Linked Immunosorbent Assays (ELISA)

All PAPS and SLE patients had the following parameters measured using an ELISA technique - TAT, F 1+2, soluble P-selectin, soluble E-selectin, D-dimer, soluble thrombomodulin, anticardiolipin antibody and anti-β_2GPI antibody (as described in section 2.4)

4.2.5 Coagulation assays

All PAPS and SLE patients had the following routine coagulation tests performed on citrated plasma samples - PT, INR, APTT (and mixing study if APTT prolonged), TT and fibrinogen (section 2.6). Lupus anticoagulant testing was performed using a DRVVT and TSVT as described in section 2.7.

4.2.6 Molecular biology tests

All patients had both Factor V Leiden (FVL) and prothrombin gene mutation (PGM) testing performed on DNA samples prepared from buffy coats as described in section 2.9.

4.3 Results

Patient characteristics

The characteristics of the twenty PAPS patients studied are shown in *Table 4.1,* and those of the thirty SLE patients in *Tables 4.2* and *4.3.* SLE patients were grouped according to the presence or absence of secondary APS. For the purposes of this thesis, SLE patients with positive APA testing but no evidence of clinical APS

manifestations were classified as APS negative, however they are also considered as a separate subgroup from those SLE patients with negative APA testing.

In the PAPS group, there were twelve female and eight male patients. Sixteen (80%) patients had a history of one or more thrombotic events. Of these, 38% were venous thromboses only, 44% were arterial and 18% were both venous and arterial. Seven of the twelve women had a history of recurrent miscarriage. Significant thrombocytopaenia (defined as a platelet count $< 100 \times 10^9$/l) had been documented in 30% of patients, either at the time of study or previously. Eighteen patients (90%) were receiving long term aspirin and/or anticoagulant therapy. They had a history of recurrent thromboembolism and/or miscarriage, or were considered to be at a significantly higher risk of thrombotic events. Of these patients, 33% were taking aspirin alone, 23% were taking warfarin or Fragmin®, and 44% were receiving both therapies. All patients satisfied the laboratory criteria for APS. The results of APA testing shown in *Table 4.1* (and the following tables) reflect the findings on the particular study day - these results will be used in any further statistical analysis, but it is important to note that the LA positivity and ACL titre in a given patient may vary quite significantly over time. Twelve patients (60%) had a positive LA test. Fifteen patients (75%) had an elevated ACL IgG level, whilst thirteen patients (65%) had an elevated ACL IgM level. Fourteen patients (70%) had antibodies to β_2GPI.

In the SLE patient group, all patients were females. Fourteen patients were classified as having secondary APS, as they had both clinical and laboratory features which fulfilled the diagnostic criteria. Of the sixteen SLE patients without APS, a total of 9 patients had positive APA testing (according to the normal cut-off criteria

Patient	Sex	Thrombosis	Miscarriage	Thrombocytopaenia[a]	Therapy	LA	ACL IgG	ACL IgM	anti-β_2GPI
1	M	Venous and arterial	-	+	Aspirin and fragmin	+	46	43	68.8
2	F	Venous and arterial	-	-	Warfarin	+	8	48	4.3
3	M	Venous	-	-	Aspirin and warfarin	-	<1	11	1.4
4	M	Venous	-	-	Warfarin	+	60	45	406
5	F	-	-	+	Aspirin	+	23	80	4.4
6	F	Arterial	-	-	Aspirin and warfarin	-	<1	10	1.4
7	M	Arterial	-	-	Aspirin	+	3	2	10
8	F	Venous and arterial	+	-	Aspirin and warfarin	+	58	35	30.9
9	F	Venous	+	+	-	+	67	7	396
10	M	Arterial	-	-	Aspirin and warfarin	-	26	3	24.2
11	F	Venous	-	-	Aspirin	+	47	<1	21.8
12	F	Venous	+	+	Warfarin	+	69	8	18.3
13	F	-	+	-	Aspirin	-	7	<1	0.42
14	F	-	-	+	-	+	50	14	615
15	M	Arterial	-	-	Aspirin and warfarin	-	35	<1	1.0
16	M	Arterial	-	-	Aspirin and warfarin	-	6	<1	47.1
17	F	Arterial	+	-	Aspirin and warfarin	+	5	68	10.9
18	F	-	+	+	Aspirin	+	58	68	3.8
19	M	Venous	-	-	Warfarin	-	16	<1	1.0
20	F	Arterial	+	-	Aspirin	-	3	15	0.69

Table 4.1 *Clinical and laboratory characteristics of 20 PAPS patients.*

F = female; M = male; + = presence or positive; – = absence or negative; ACL = anticardiolipin level (in either GPL or MPL units) normal range < 5 GPL or < 5 MPL units; LA = lupus anticoagulant; anti-β_2GPI = anti-β_2GPI % concentration (normal range < 2.88%) ; [a] previously documented platelet count of < 100×10^9/l.

Patient	Thrombosis	Miscarriage	Thrombocytopaenia[a]	Therapy	LA	ACL IgG	ACL IgM	ACL anti-β_2GPI
1	-	–	+	Aspirin and immunosuppressive	–	20	49	18
2	Venous	+	+	Warfarin and immunosuppressive	+	14	<1	0.7
3	Venous	+	–	Warfarin	–	9	<1	6.3
4	-	–	+	Aspirin and immunosuppressive	+	88	3	39.5
5	Arterial	+	–	Warfarin and immunosuppressive	–	92	20	106.3
6	-	+	–	Aspirin and immunosuppressive	–	16	3	6.6
7	-	–	+	Immunosuppressive	+	12	<1	10.3
8	Arterial	–	–	Warfarin and immunosuppressive	+	50	<1	12.3
9	Arterial	+	–	Aspirin and immunosuppressive	+	20	4	1.8
10	-	–	+	Aspirin and immunosuppressive	–	28	77	3.3
11	Venous and arterial	–	–	Warfarin	+	88	<1	110
12	-	–	+	Aspirin and immunosuppressive	–	16	43	5
13	Venous and arterial	+	–	Warfarin and immunosuppressive	+	83	8	42.3
14	Venous	–	–	Warfarin and immunosuppressive	–	16	<1	9.1

Table 4.2 *Clinical and laboratory characteristics of 14 SLE patients with secondary APS.*

F = female; M = male; + = presence or positive; – = absence or negative; ACL = anticardiolipin level (in either GPL or MPL units) normal range < 5 GPL or < 5 MPL units; LA = lupus anticoagulant; anti-β_2GPI = anti-β_2GPI % concentration (normal range < 2.88%) ; [a] previously documented platelet count of < 100×10^9/l.

Patient	Thrombosis	Miscarriage	Thrombocytopaenia[a]	Therapy	LA	ACL IgG	ACL IgM	anti-β_2GPI
1	–	–	–	–	–	2	<1	0.95
2	–	–	–	–	–	2	<1	0.45
3	–	–	–	Immunosuppressive	–	8	2	0.61
4	–	–	–	Immunosuppressive	+ (weak)	<1	4	1.1
5	–	+	–	Aspirin and immunosuppressive	–	3	<1	0.4
6	–	–	–	Immunosuppressive	–	2	3	1.1
7	–	–	–	–	–	2	<1	2.4
8	Arterial	–	–	Warfarin and immunosuppressive	–	5	1	0.92
9	–	–	–	–	–	6	7	2.6
10	–	+	–	–	–	3	5	0.57
11	–	–	–	Aspirin and immunosuppressive	–	4	5	0.77
12	–	–	–	–	+ (weak)	2	4	0.45
13	–	–	–	–	+	2	28	0.98
14	–	–	–	Immunosuppressive	–	2	<1	0.79
15	–	+	–	Immunosuppressive	–	7	3	0.62
16	–	–	–	Immunosuppressive	–	1	3	2

Table 4.3 *Clinical and laboratory characteristics of 16 SLE patients without secondary APS (includes those with positive APA testing)*

F = female; M = male; + = presence or positive; – = absence or negative; ACL = anticardiolipin level (in either GPL or MPL units) normal range < 5 GPL or < 5 MPL units; LA = lupus anticoagulant; anti-β_2GPI = anti-β_2GPI % concentration (normal range < 2.88%) ; [a] previously documented platelet count of < 100×10^9/l.

for positive ACL and LA testing). However, in eight of these patients, the LA was weak, and/or the ACL titre was < 10. None of these patients had antibodies to β_2GPI. A total of nine SLE patients had a history of thrombosis, and eight of these had APA. Nine patients had miscarriages, and of these, 3 occurrred in women without APS. Patients with thrombocytopaenia as the only manifestation of APS were included in the secondary APS group if they also had positive APA testing, and notably, all six thrombocytopaenic SLE patients had APA. The platelet count was significantly lower in SLE APA positive patients than in SLE APA negative patients (p=0.042), however there was no significant difference in platelet count between controls, PAPS patients and SLE patients as a group. Sixteen patients were receiving long term aspirin or anticoagulant therapy. The majority of these were in the APS group. A total of twenty one patients were receiving immunosuppressive therapy, which usually consisted of prednisolone and/or azathioprine. At the time of the study, ten patients had a positive LA test (weak in two of these patients). Seventeen patients had an elevated ACL IgG level, whilst seven patients had an elevated ACL IgM level. Twelve of the fourteen SLE patients with secondary APS had antibodies to β_2GPI, whilst none of the SLE patients without APS had these antibodies.

Twenty healthy volunteers were used as control subjects for the platelet studies. There were twelve females and eight males, and their ages ranged between 21 and 45 years.

Results summary

Median values (with 5th and 95th percentiles) for all assays performed in controls, PAPS and SLE patients are listed in *Table 4.4.* Only median values will be referred to in the following text.

Parameter	Controls	PAPS	SLE
Platelet count ($\times 10^9$/l)	220 (166, 285)	217 (85, 387)	199 (143, 305)
CD62p (%)	1.2 (0.5, 2.5)	1.3 (0.4, 5.1)	1.6 (0.5, 4.8)
CD63 (%)	3.0 (1.3, 2.1)	5.2 (2.1, 8.9)	3.1 (1.6, 7.2)
Annexin V (%)	3.0 (1.4, 4.6)	3.2 (1.7, 6.6)	3.1 (1.9, 9.1)
PAC-1 (%)	3.7 (2.5, 6)	7.5 (2.7, 39.9)	3.6 (2.6, 8.0)
Microparticles ($\times 10^6$/ml)	0.35 (0.17, 0.87)	0.45 (0.14, 1.41)	0.40 (0.16, 2.32)
Platelet-granulocytes (%)	3.5 (2.7, 4.3)	3.7 (1.6, 5.1)	4.6 (2.8, 8.3)
Platelet-monocytes (%)	5.5 (3.9, 7.7)	7.6 (3.8, 10.8)	9.2 (5.1, 14.7)
Platelet-lymphocytes (%)	3.0 (2.1, 3.8)	2.9 (1.8, 4.4)	3.7 (2.3, 5.4)
soluble P-selectin (ng/ml)	18.8 (8.5, 36.8)	41.4 (16.0, 76.5)	32.1 (22.1, 53.8)
TAT (μg/ml)	2.4 (1.1, 4.8)	2.1 (1.0, 6.2)	2.4 (0.9, 9.1)
F 1+2 (nmol/l)	0.8 (0.4, 1.6)	0.5 (0.1, 1.7)	0.7 (0.8, 1.4)
D-Dimers (ng/ml)	0.6 (0.0, 25.3)	5.9 (0.0, 42.8)	0.0 (0.0, 47.5)
soluble E-selectin (ng/ml)	32.8 (20.4, 57.4)	48.5 (19.7, 95.3)	46.0 (24.5, 70.5)
Thrombomodulin (ng/ml)	50.9 (27.2, 69.1)	81.8 (35.1, 134.3)	57.3 (27.9, 152.5)

Table 4.4 *Median values (with 5^{th} and 95^{th} percentiles) for all assays performed in controls, PAPS patients and SLE patients.*

Expression of platelet degranulation markers

Results for the percentage of platelets expressing CD62p and CD63 are shown in *Figures 4.1 and 4.2* respectively. The median values for CD62p expression were similar amongst the three groups - they were 1.2% for the control group, 1.3% for PAPS patients, and 1.6% for SLE patients. However, there were some individuals in both patient groups who had relatively high levels of CD62p expression. There was a significant difference in median CD63 expression between PAPS and controls ($p=0.007$) and PAPS and SLE patients ($p=0.002$), but not between SLE patients and controls. Median values were 3% for controls, 5.2% for PAPS, and 3.1% for SLE. Further analysis of results demonstrated that median CD63 expression was significantly higher in PAPS patients compared with both SLE patients with secondary APS ($p=0.03$), and SLE patients without secondary APS ($p=0.002$). There was no significant difference in median CD62p or CD63 expression between SLE patients with APS and SLE patients without secondary APS. In both patient groups, there was no correlation between the values obtained for CD62p and CD63 expression, nor was there any correlation between titre of ACL or anti-β_2GPI antibodies and expression of these platelet degranulation markers.

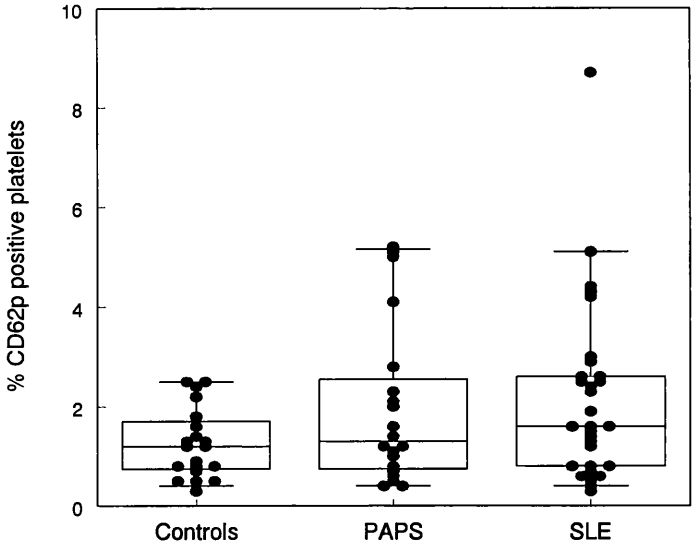

Figure 4.1 *% platelets expressing CD62p in controls (n=20), PAPS (n=20) and SLE patients (n=30).*

Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p=ns (all groups)

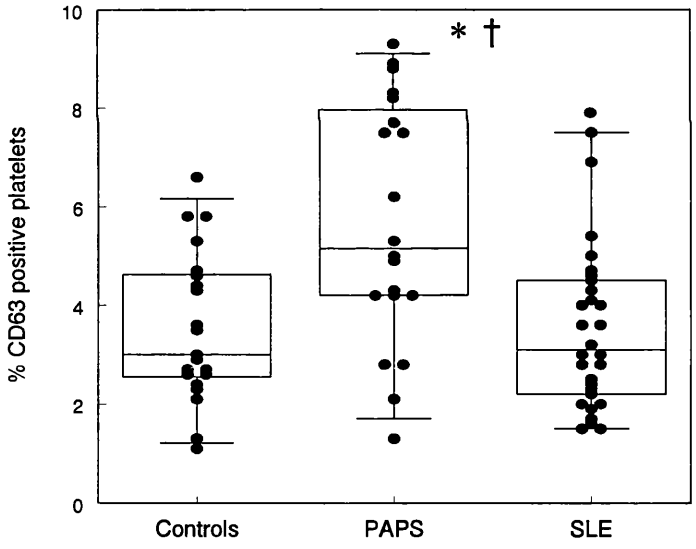

Figure 4.2 *% platelets expressing CD63 in controls (n=20), PAPS (n=20) and SLE patients (n=30).*

Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.0066 (PAPS *vs* Cont), † p = 0.0021 (PAPS *vs* SLE), p=ns (SLE *vs* Cont)

PAC-1 and annexin V binding to platelets

Results for the percentage of platelets binding PAC-1 and annexin V are shown in *Figures 4.3 and 4.4* respectively.

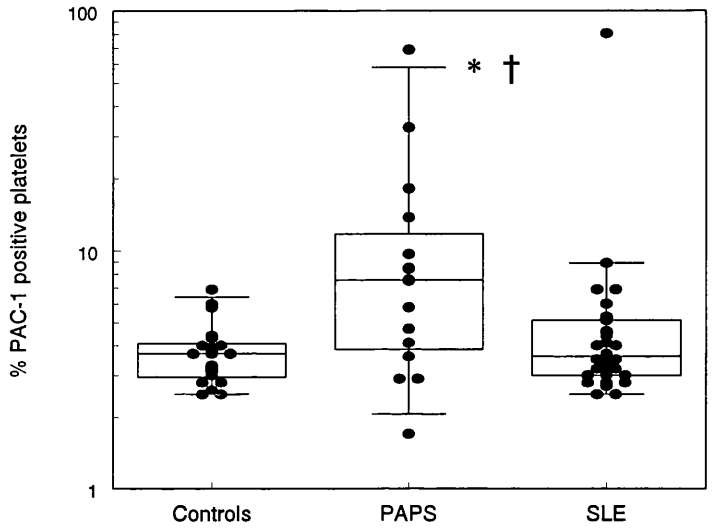

Figure 4.3 *% platelets binding PAC-1 in controls (n=20), PAPS (n=17) and SLE patients (n=30).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.007 (PAPS *vs* Cont), † p = 0.015 (PAPS *vs* SLE), p=ns (SLE *vs* Cont)

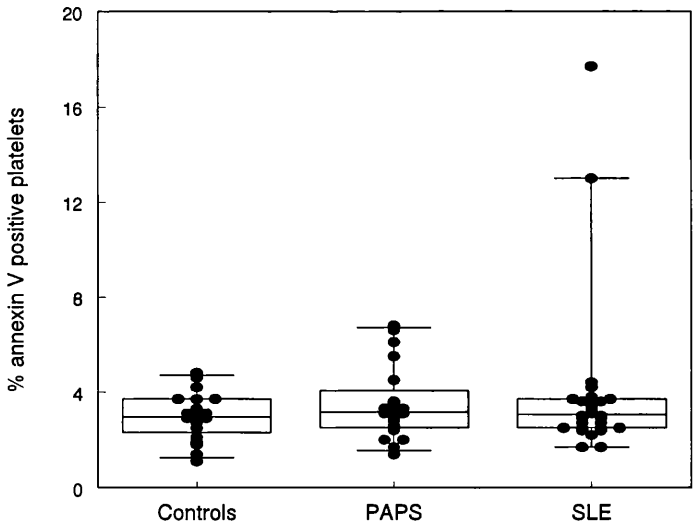

Figure 4.4 *% platelets binding annexin V in controls (n=20), PAPS (n=20) and SLE patients (n=30).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p = ns (all groups)

The median values for PAC-1 binding were 3.7% in controls, 7.5% in PAPS patients, and 3.6% in SLE patients. There was a significant difference in results between PAPS patients and controls (p=0.007), as well as PAPS and SLE patients (p=0.015). The difference between PAPS patients and SLE patients with secondary APS was not significant for this parameter. Notably, some patients in both groups had extremely elevated levels of PAC-1 binding.

The median values for annexin V binding were 3.0% in controls, 3.2% in PAPS, and 3.1% in SLE patients. Although there was no significant difference between the median values for the three groups, some patients (in particular those with SLE) had high levels of annexin V binding.

There was no correlation between either PAC-1 or annexin-V binding and the presence or absence of ACL antibodies, LA activity or antibodies to β_2GPI. In those patients with ACL and anti-β_2GPI antibodies, there was no correlation between the level of PAC-1 or annexin V binding and the antibody titre. There was no difference in either PAC-1 or annexin V binding between SLE patients with APS and SLE patients without APS.

In the PAPS patient group, there was a positive correlation between platelet CD62p expression and PAC-1 binding to platelets (rs=0.70, p=0.002). This was not the case with the SLE patient group however.

Platelet microparticles

Results for numbers of microparticles/ml plasma are shown in *Figure 4.5.* The median values for numbers of microparticles were 0.35 $\times 10^6$ in controls, 0.45 $\times 10^6$ in PAPS patients, and 0.40 $\times 10^6$ in SLE patients. There was no significant difference in the median values between the three groups, although some SLE patients had very high numbers of microparticles. In the PAPS patient group there was a positive correlation between absolute platelet count and numbers of microparticles/ml plasma (rs=0.69, p=0.0009). This was not the case with the SLE patient group however.

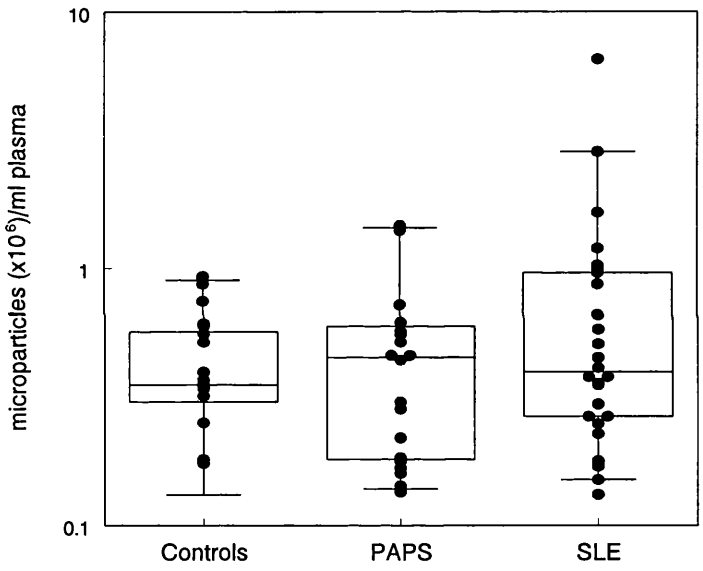

Figure 4.5 *Numbers of microparticles/ml plasma in controls (n=20), PAPS (n=20) and SLE patients (n=30).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p =ns (all groups)

Platelet-leukocyte complexes

Results for the percentage of platelet-granulocyte complexes, platelet-monocyte complexes, and platelet-lymphocyte complexes are illustrated in *Figures 4.6, 4.7, and 4.8* respectively. The median values for platelet-granulocyte complexes were 3.5% in controls, 3.7% in PAPS patients, and 4.6% in SLE. These values were significantly

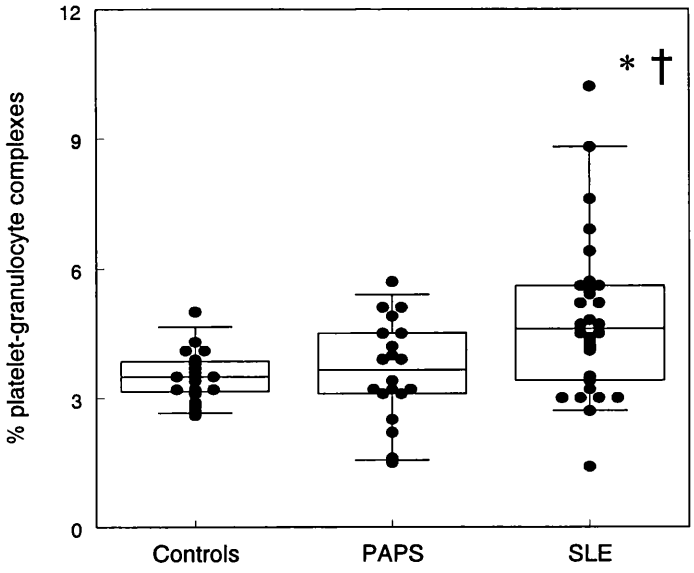

Figure 4.6 *% platelet-granulocyte complexes in controls (n=20), PAPS (n=20) and SLE patients (n=30).*

Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.0029 (SLE *vs* Cont), † p = 0.0251 (SLE *vs* PAPS), p=ns (PAPS *vs* Cont)

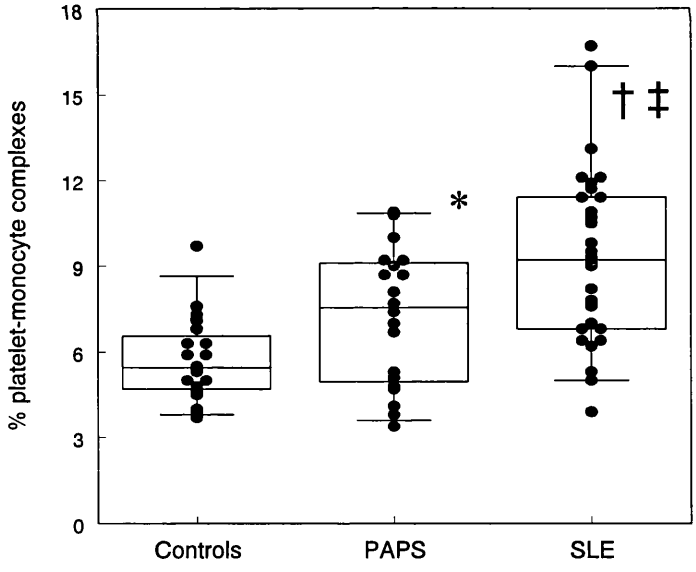

Figure 4.7 *% platelet-monocyte complexes in controls (n=20), PAPS (n=20) and SLE patients (n=30).*

Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.0482 (PAPS *vs* Cont), † p<0.0001 (SLE *vs* Cont), ‡ p = 0.0189 (SLE *vs* PAPS)

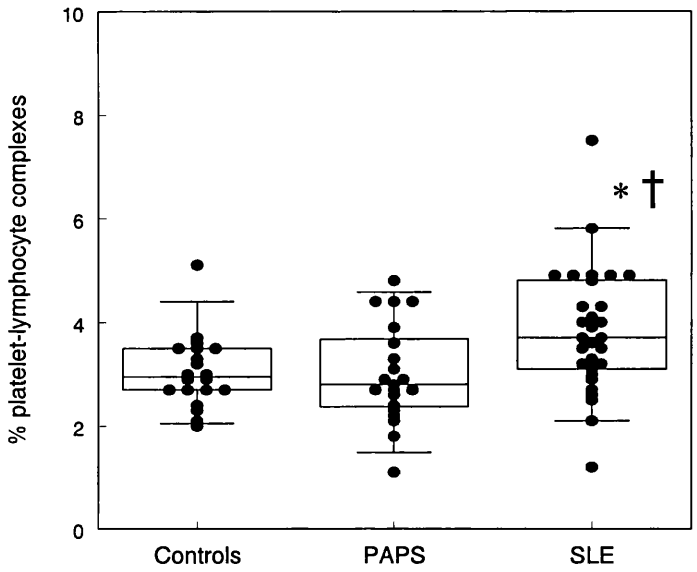

Figure 4.8 *% platelet-lymphocyte complexes in controls (n=20), PAPS (n=20) and SLE patients (n=30).*

Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.0056 (SLE *vs* Cont), † p = 0.0122 (SLE *vs* PAPS), p=ns (PAPS *vs* Cont)

higher in SLE patients when compared to both controls (p=0.003) and PAPS (p=0.025). There was no significant difference between SLE patients with APS and SLE patients without APS. Median values for platelet-monocyte complexes were 5.5% in controls, 7.6% in PAPS, and 9.2% in SLE patients. There was a signficant difference in percentage platelet-monocyte complexes between controls and PAPS (p=0.048), controls and SLE (p<0.0001) and PAPS and SLE (p=0.019). There was no significant difference between SLE patients with APS and SLE patients without APS. The median values for percentage platelet-lymphocyte complexes were 3.0% in controls, 2.9% in PAPS, and 3.7% in SLE. The percentage of platelet-lymphocyte complexes was significantly higher in SLE patients when compared to both controls (p=0.006) and PAPS (p=0.01). The median levels were also significantly higher in SLE patients without APS compared to those with APS (p=0.003).

In the PAPS patient group, there was a positive correlation between absolute platelet count and percentage platelet-granulocyte complexes (rs=0.78, p<0.0001); platelet-monocyte complexes (rs=0.62, p=0.004); and platelet-lymphocyte complexes (rs=0.58, p=0.007). A positive correlation between platelet-granulocyte and both platelet-monocyte complexes (rs = 0.68, p = 0.001) and platelet-lymphocyte complexes (rs = 0.77, p < 0.0001) was also noted.

In the SLE patient group there was a positive correlation between annexin V binding to platelets and percentage platelet-monocyte complexes (rs=0.47, p=0.019). There was also a positive correlation between platelet count and percentage platelet-granulocyte complexes (rs=0.54, p = 0.002); and platelet-lymphocyte complexes (rs= 0.49, p=0.006), but not platelet-monocyte complexes nor numbers of microparticles. A positive correlation between platelet-granulocyte and both platelet-monocyte complexes (rs = 0.79, p < 0.0001) and platelet-lymphocyte complexes (rs = 0.79, p < 0.0001) was also noted. There was no correlation between platelet-leukocyte complexes and any of the other flow cytometric platelet activation markers.

Platelet-leukocyte complexes in RA patients

Since an increase in platelet-leukocyte complexes was found particulary in patients with SLE, ten patients with RA (who were negative for LA, ACL and anti-β_2GPI antibodies) were studied in order to determine whether this increase may be found in patients with autoimmune disorders in general. The results are shown in *Figures 4.9, 4.10, and 4.11.* The difference in percentage platelet-granulocyte complexes between RA patients and controls was significant (p=0.0008), as was the difference in platelet-monocyte complexes (p=0.002). Although the difference in percentage platelet-lymphocyte complexes failed to reach statistical significance, some RA patients had elevated levels of platelet-lymphocyte complexes. It is important to note that the RA patients had a significantly higher platelet count when compared to controls (p=0.0003), PAPS patients (p=0.012), and SLE patients (p=0.0002).

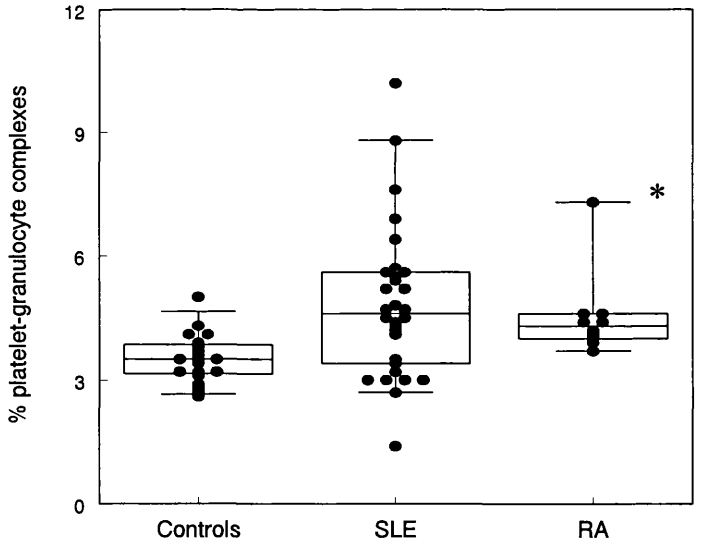

Figure 4.9 *% platelet-granulocyte complexes in controls (n=20), SLE (n=30) and RA patients (n=10).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.0008 (RA *vs* Cont)

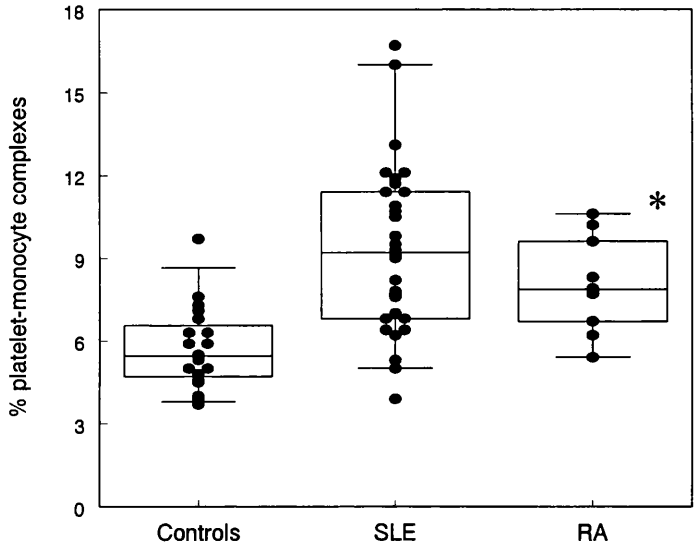

Figure 4.10 *% platelet-monocyte complexes in controls (n=20), SLE (n=30) and RA patients (n=10).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.0014 (RA *vs* Cont)

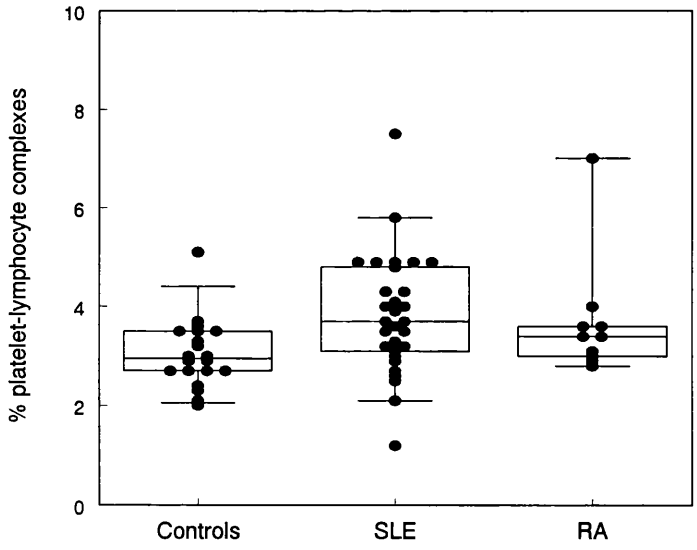

Figure 4.11 *% platelet-lymphocyte complexes in controls (n=20), SLE (n=30) and RA patients (n=10).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p=ns (RA *vs* Cont)

Patients receiving aspirin therapy

In the PAPS group, fourteen patients were receiving aspirin, and in the SLE group, eight patients were on chronic daily aspirin therapy. There was no significant difference in any of the platelet activation markers cited above, between patients on aspirin and those not on aspirin for both patient groups. This is in contrast to the results from Chapter 3 whereby PAPS patients on aspirin had significantly lower values for CD63 expression than those not on aspirin.

Platelet activation markers and thrombosis

In both PAPS and SLE patient groups, there was no significant difference in any of the platelet activation markers measured between patients with a history of thrombosis and those without. Unlike the findings in Chapter 3, there was no association between the presence of antibodies to β_2GPI, and a history of thrombosis in either the PAPS or SLE with secondary APS groups.

Despite the fact that there was no general correlation between all of the platelet activation markers in individual patients, there was one particular patient who had very high levels of multiple markers. This patient had SLE with secondary APS and a history of venous thrombosis, recurrent miscarriage, and immune mediated thrombocytopaenia for which a splenectomy had been performed. Levels of CD62p expression, PAC-1 binding, annexin-V binding, and percentage platelet-leukocyte complexes (of all three types), were all significantly elevated, and amongst the highest levels detected in any of the patients studied.

Plasma soluble P-selectin

When compared to controls, median levels of plasma soluble P-selectin were significantly higher in both PAPS patients ($p=0.0005$) and SLE patients ($p=0.0002$) as seen in *Figure 4.12.* The respective values were 18.8 ng/ml in controls, 41.4 ng/ml in PAPS, and 32.1 ng/ml in SLE patients. The difference between soluble P-selectin levels in PAPS and SLE patients was also significant ($p<0.05$). In the PAPS patient group, there were weak positive correlations between levels of soluble P-selectin and both platelet CD63 expression ($rs=0.45$, $p<0.05$), and percentage platelet-monocyte complexes ($rs=0.48$, $p=0.03$). This was not the case with SLE patients however. There was no significant difference in P-selectin levels between SLE patients with APS and those without APS.

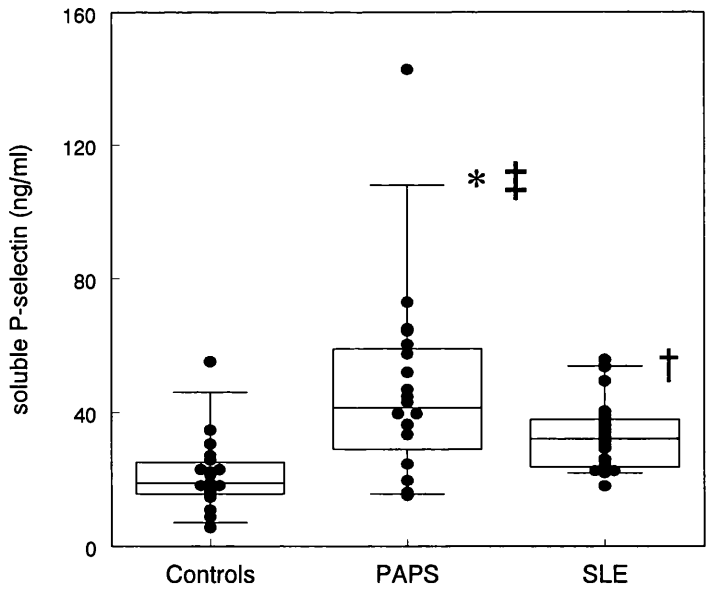

Figure 4.12 *Plasma soluble P-selectin levels in controls (n=20), PAPS (n=20) and SLE patients (n=30).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.0005 (PAPS *vs* Cont), † p = 0.0002 (SLE *vs* Cont), ‡ p = 0.0455 (SLE *vs* PAPS)

Thrombin generation markers and D-dimer formation

Results for the levels of TAT, F 1+2 and D-dimers are shown in *Figures 4.13, 4.14 and 4.15.* There was no significant difference in median TAT levels between the groups. The values were 2.35 μg/ml in controls, 2.08 μg/ml in PAPS patients, and 2.37 μg/ml in SLE patients. Median values for F 1+2 were 0.80 nmol/l in controls, 0.45 nmol/l in PAPS patients, and 0.70 nmol/l in SLE patients. The median F 1+2 level in PAPS was lower than that in controls, however the difference just failed to reach statistical significance (p=0.05). Median D-dimer levels were 0.60 ng/ml in controls, 5.94 ng/ml in PAPS patients, and 0.0 ng/ml in SLE patients. The difference in D-dimer levels between PAPS and SLE patients was significant (p=0.04). There was a significant positive correlation between TAT and F 1+2 levels in PAPS patients (Spearman Rank Correlation Coefficient [rs]=0.61, p=0.004) and also in SLE patients (rs=0.62, p=0.0003). Both median levels of TAT and F 1+2 were significantly higher in SLE patients without APS compared to SLE patients with APS (p=0.03 and p=0.02 respectively).

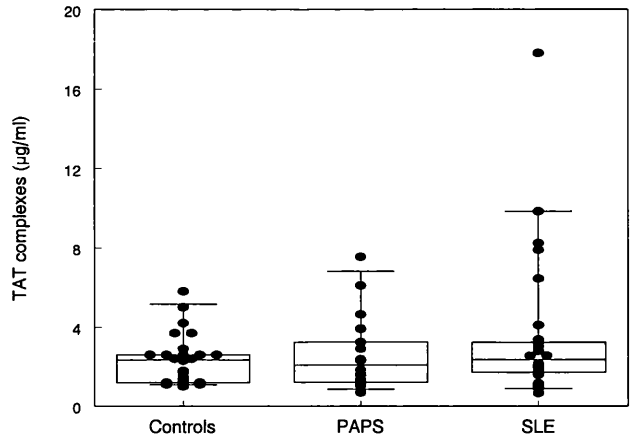

Figure 4.13 *Plasma levels of TAT complexes in controls (n=26), PAPS (n=20) and SLE patients (n=30).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p =ns (all groups)

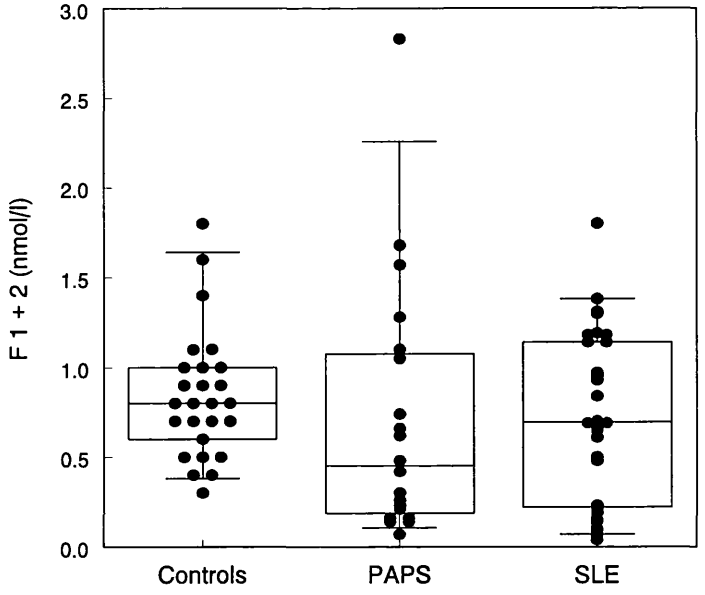

Figure 4.14 *Plasma levels of F 1 + 2 in controls (n=26), PAPS (n=20) and SLE patients (n=30).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p =ns (all groups)

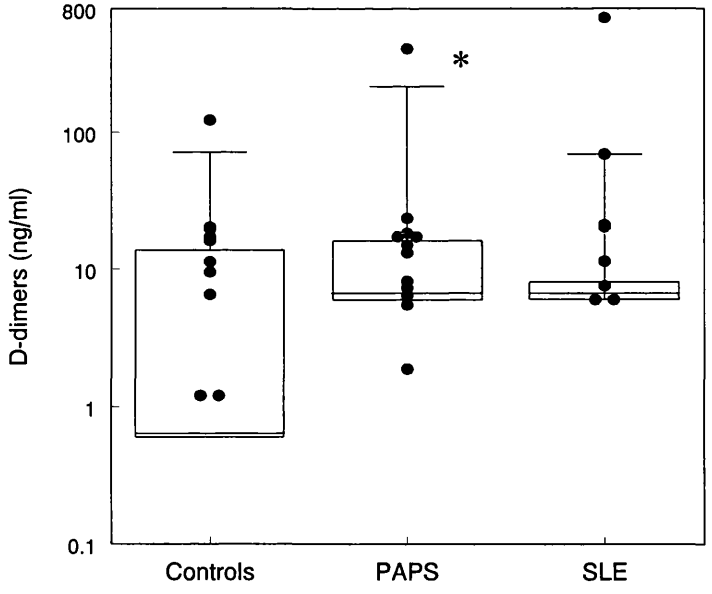

Figure 4.15 *Plasma D-dimer levels in controls (n=20), PAPS (n=20) and SLE patients (n=30).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. p = 0.0396 (PAPS *vs* SLE), p = ns (PAPS *vs* Cont; SLE *vs* Cont)

Patients receiving oral anticoagulant therapy

In the PAPS and SLE patient groups, 19 patients in total were receiving long term oral anticoagulant therapy at the time of the study. *Figures 4.16 and 4.17* demonstrate the results of TAT and F 1+2 levels in all patients on warfarin in comparison to patients not on warfarin, and controls.

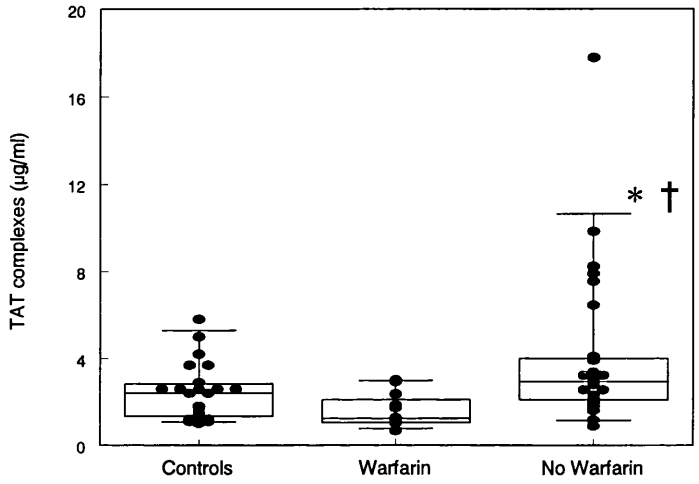

Figure 4.16 *Plasma levels of TAT complexes in controls (n=26), and in patients either receiving warfarin (n=19) or no warfarin (n=31).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p = 0.04 (No Warf *vs* Cont), † p = 0.0006 (Warf *vs* No Warf)

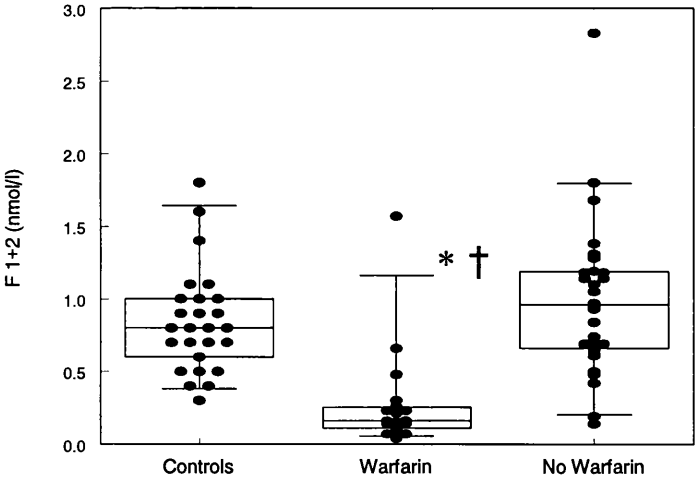

Figure 4.17 *Plasma levels of prothrombin fragment 1 + 2 in controls (n=26) and in patients either receiving warfarin (n=19) or no warfarin (n=31).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. * p <0.0001 (Cont *vs* Warf), † p < 0.0001 (Warf *vs* No Warf)

The median values for TAT were 2.35 μg/ml in controls, 1.37 μg/ml in patients on warfarin, and 2.82 μg/ml in patients not on warfarin. The difference in TAT levels between warfarinised and non-warfarinised patients was significant (p=0.0006), as was the difference between non-warfarinised patients and controls (p=0.04). Corresponding values for F 1+2 levels were 0.80 nmol/l in controls, 0.16 nmol/l in patients on warfarin, and 0.96 nmol/l in patients not on warfarin. The difference in F 1+2 levels between warfarinised and non-warfarinised patients was significant (p<0.0001), as was the difference between warfarinsed patients and controls (p<0.0001). There was no significant difference in D-dimer levels between warfarinised and non-warfarinised patients.

Correlation between INR and TAT and F 1+2 levels

There was a significant negative correlation between a patients INR and TAT levels (rs= –0.63, p<0.0001) and a patients INR and F 1+2 levels (rs = –0.82, p<0.0001) as seen in *Figure 4.18*. In contrast to the finding in Chapter 3, no warfarinised patients had elevated levels of either TAT or F 1+2.

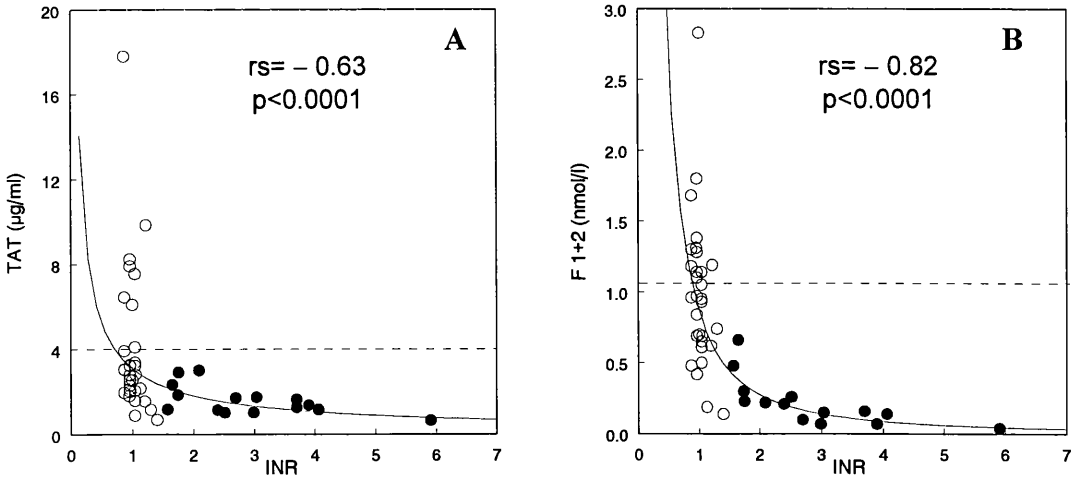

Figure 4.18 A and B *Correlation between INR and TAT, and INR and F 1+2 levels all patients.*

Dotted horizontal line indicates upper limit of kit assay normal range. Open circles indicate non-warfarinised patients; closed circles indicate warfarinised patients.

Thrombin generation and platelet activation

In both groups of patients studied, there was no significant difference in the levels of platelet activation markers between patients on warfarin and those not on warfarin. In particular, warfarin did not result in a significant reduction in platelet activation.

Endothelial cell 'activation' markers

Results of soluble E-selectin and thrombomodulin levels in controls and patients are shown in *Figures 4.19 and 4.20.* The median values for soluble E-selectin were 32.8 ng/ml in controls, 48.5 ng/ml in PAPS patients, and 46.0 ng/ml in SLE patients. The difference in median soluble E-selectin levels between PAPS patients and controls was significant (p=0.02) as was the difference between SLE patients and controls (p=0.002). There was no significant difference in E-selectin levels between SLE patients with APS and SLE patients without APS.

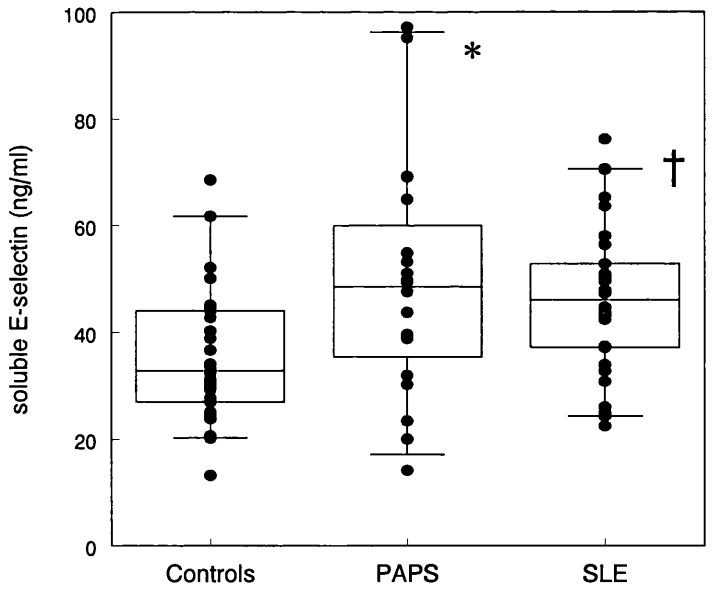

Figure 4.19 *Plasma soluble E-selectin levels in controls (n=30), PAPS (n=20) and SLE patients (n=30).*

Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. *p = 0.02 (PAPS *vs* Cont), † p = 0.002 (SLE *vs* Cont), p=ns (PAPS *vs* SLE)

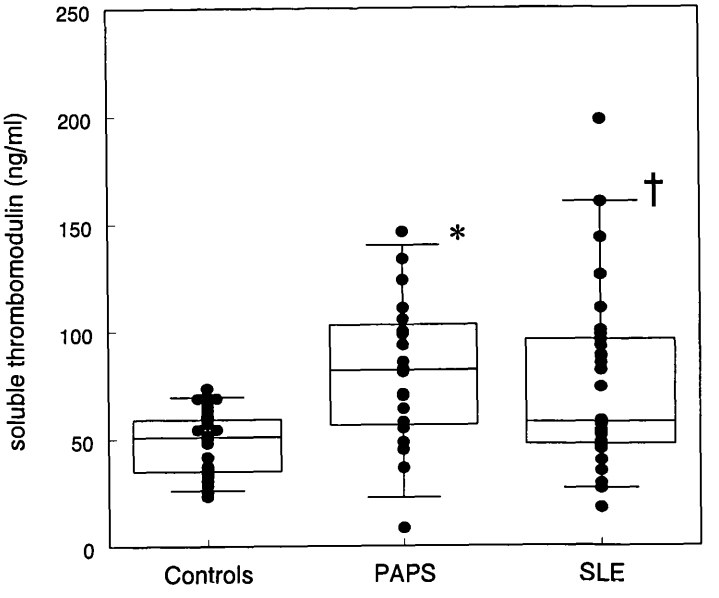

Figure 4.20 *Plasma thrombomodulin levels in controls (n=34), PAPS (n=20) and SLE patients (n=30).*

Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. *p = 0.0001 (PAPS *vs* Cont), † p = 0.01 (SLE *vs* Cont), p=ns (PAPS *vs* SLE)

Thrombomodulin levels were significantly higher in PAPS and SLE patients compared to controls (p=0.0001 and p=0.01 respectively). Median values were 50.9 ng/ml in controls, 81.8 ng/ml in PAPS patients, and 57.3 ng/ml in SLE patients. Median TM levels were significantly higher in SLE patients with APS compared to SLE patients without APS. Since TM levels are known to be increased in patients with renal impairment, results were interpreted with this in mind. Levels of serum creatinine (which were routinely performed on most patients) are shown in *Figure 4.21.* The normal range for creatinine is 62-133 μmol/l. In PAPS patients, the median value (with 5th and 95th percentiles) was 102 μmol/l (67μmol/l, 137 μmol/l) and in SLE patients, the corresponding values were 70 μmol/l (56 μmol/l, 118 μmol/l). The difference between median values in PAPS and SLE patients was significant (p=0.03). Interestingly, SLE patients with APS had a significantly higher

median level of serum creatinine (75 μmol/l) compared to SLE patients without APS (62 μmol/l, p=0.01). Two PAPS patients and one SLE patient had creatinine levels which were above the normal range.

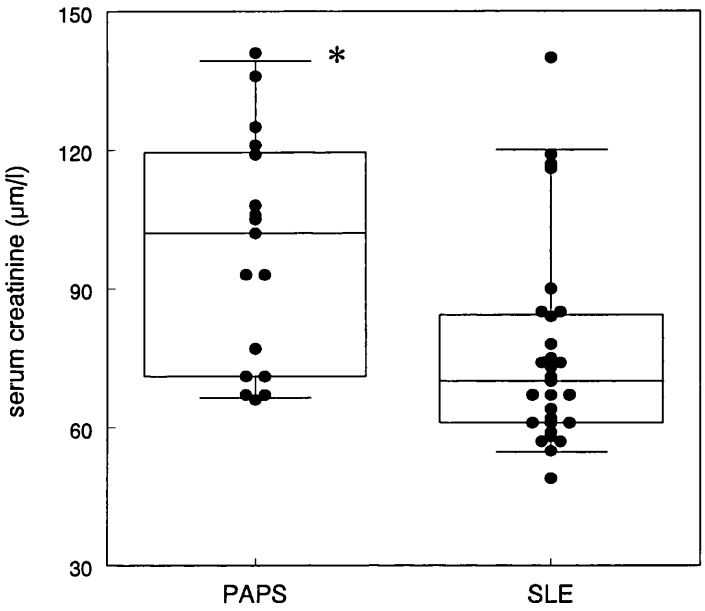

Figure 4.21 *Serum creatinine levels in PAPS patients (n=17) and SLE patients (n=29).*
Horizontal line indicates median, box the 25th to 75th percentile, and bars the 5th and 95th percentile. *p=0.03

In PAPS patients there was a significant positive correlation between serum creatinine and both TM levels (rs=0.57, p=0.02), and E-selectin levels (rs=0.65, p=0.005). In SLE patients, there was a significant positive correlation between serum creatinine and TM (rs=0.65, p=0.0001) but not E-selectin. There was no correlation between levels of soluble E-selectin and thrombomodulin in any of the patient groups. For both of these parameters, there was no significant difference in median levels between patients on aspirin or no aspirin, and patients on warfarin or no warfarin.

Correlation between platelet activation, thrombin generation and endothelial activation markers

In the PAPS patient group, there were no significant correlations between markers of platelet activation, thrombin generation and endothelial cell activation (apart from those mentioned previously). In the SLE patient group, there was a significant positive correlation between percentage platelet-granulocyte complexes and both TAT and F 1+2 levels (rs=0.52, p=0.003 and rs=0.39, p=0.04 respectively). There was also a significant positive correlation between percentage platelet-monocyte complexes and both TAT and F 1+2 levels (rs=0.50, p=0.005 and rs=0.37, $p<0.05$ respectively).

FVL and PGM results

All patients had FVL and PGM testing performed. Two patients (one with PAPS, one with SLE) were found to be heterozygote for the FVL mutation, whilst none of the fifty patients studied had the PGM detected. The PAPS patient with FVL had a history of arterial thrombosis only, and did not have a more severe form of thrombotic disease compared with the other patients. The SLE patient with FVL had no thormbotic history, was APA negative, but did have a history of one miscarriage.

4.4 Discussion

Numerous publications have demonstrated evidence of *in vivo* platelet activation in patients with APS, and this was supported by the findings of increased platelet CD63 expression and levels of plasma soluble P-selectin in Chapter 3. Most recent studies have examined the platelet degranulation markers CD62p and CD63, however platelet activation is a complex process, hence it is possible that measuring degranulation

markers alone may limit the ability to detect platelet activation under all circumstances. Since platelets are likely to become activated by different agonists in different clinical situations, the pattern of platelet surface activation may vary, and therefore it is probably best to employ a panel of activation-dependent antibodies. An advantage of some antibodies (i.e. PAC-1), is that they detect platelets that have been stimulated with agonists such as ADP under conditions where the surface expression of secretion dependent markers such as CD62p is limited. Also, numerous flow cytometric methods which examine other aspects of platelet actvation (such as aminophospholipid exposure, microparticle formation, platelet-leukocyte complexes) have been developed recently, and these were used to study a further group of PAPS patients as well as SLE patients with and without secondary APS.

In this investigation, there was no signficant difference in median values for CD62p expression amongst the three groups studied. However, median CD63 expression was significantly higher in PAPS patients compared to controls and SLE patients. These findings are in concordance with the results from Chapter 3, and their significance has been discussed in detail in the previous chapter. An interesting finding from the current study was the significantly higher median CD63 expression found in PAPS patients compared to the group of SLE patients with secondary APS. One possible reason for this result may be that the majority of SLE patients were receiving immunosuppressive therapy at the time of the study which could potentially ameliorate a platelet-activating/degranulating action of APA.

The binding of the antibody PAC-1 (which detects a conformational change in the platelet GP IIb/IIIa complex upon activation) was found to be significantly increased in PAPS patients, although one patient with SLE and secondary APS had

the highest level of PAC-1 binding (81%). In comparison to the results for CD63 expression, there was no significant difference in levels of PAC-1 binding between PAPS patients and SLE patients with secondary APS. This may be because PAC-1 binding is a more 'sensitive' index of platelet activation than the expression of degranulation markers. Indeed, a study which examined activated platelets in patients undergoing thrombolysis following acute myocardial infarction, found high levels of PAC-1 binding despite low levels of platelet CD62p expression (Bihour *et al*, 1995).

Although there was no significant difference in median levels of annexin V binding between controls, PAPS and SLE, some individual patients had relatively high values. The binding of annexin V to platelets reflects aminophospholipid exposure, and it has been demonstrated from *in vitro* experiments that strong agonists such as calcium ionophore A23187 and collagen and thrombin can increase the number of annexin V binding sites on platelets (Thiagarajan & Tait 1990). Under normal resting conditions, it is unlikely that significant aminophospholipid exposure would occur, and so this may partly explain the findings of the current study. Another possibility is that highly 'reactive' PS expressing platelets are rapidly cleared from the circulation. It should also be considered that APA may interfere with the binding of annexin V to PS. One study found that APA IgG's reduce the quantity of annexin V bound to phospholipid bilayers, and this reduction was dependent on the presence of β_2GPI (Rand *et al*, 1998).

Microparticle numbers were not significantly increased in PAPS or SLE patients, but there were several patients with elevated levels. The highest number of microparticles (6.5×10^6/ml plasma) was found in an SLE patient without secondary APS and no laboratory evidence of APA. Similar to the findings for annexin V

binding, it may be that microparticle numbers are not increased in resting conditions, since it is known that there is only extensive *in vitro* formation of platelet microparticles following exposure to calcium ionophore A23187, collagen plus thrombin or complement C5b-9 (Zwaal *et al*, 1992). Alternatively, microparticles are also probably rapidly cleared from the circulation. Increased numbers of microparticles have been reported in patients with thrombotic thrombocytopaenic purpura and haemolytic uraemic syndrome (Galli *et al*, 1996b), as well as in the systemic and especially pericardial blood of patients undergoing cardiopulmonary bypass (Nieuwland *et al*, 1997). There have been very few reports of microparticle numbers in APS - one paper which used a whole blood method to measure percentage microparticles, found a significant increase in the mean percentage of microparticles in 11 APA patients (10%) compared with normals (5.5%) (Galli *et al*, 1993). However, there was no correlation between microparticle percentages, platelet number, ACL titre, or degree of prolongation of phospholipid dependent coagulation tests by LA.

Platelet-leukocyte complexes were generally increased in SLE and PAPS patients in comparison to controls. Although overall there was a positive correlation between the absolute platelet count and percentage platelet-leukocyte complexes, differences in platelet counts alone cannot adequately explain these findings since there was no significant difference in platelet count between SLE, PAPS and controls. The percentages of platelet-granulocyte and platelet-lymphocyte complexes were signficantly higher in SLE patients compared to PAPS and controls, whereas platelet-monocyte complexes were significantly elevated in both PAPS and SLE patients, although levels in SLE were much higher than those in PAPS. It is becoming

increasingly appreciated that leukocytes play a role in haemostasis and thrombosis and one way in which they may do so is through the formation of such platelet-leukocyte complexes. Platelet-monocyte interactions may accelerate generation of tissue factor by activated monocytes (Silverstein & Nachman 1987). A number of studies have examined platelet-leukocyte aggregates using flow cytometric techniques in different clinical conditions. One group found evidence of increased platelet-leukocyte aggregates in SLE and APS patients, and suggest that these aggregates may be a possible source of microembolic signals as detected by transcranial doppler (Specker *et al*, 1998). Another group have demonstrated that the plasma from patients with thrombotic thrombocytopaenic purpura was able to induce leukocyte-bound platelet aggregates (Valant *et al*, 1998). Although it is known that activated platelets may bind to leuckocytes (primarily through P-selectin), it is unlikely that this is the only mechanism for their formation. Evidence of platelet activation was found more often in PAPS than SLE patients, yet SLE patients generally had higher percentages of circulating platelet-leukocyte complexes. It may be that platelet-leukocyte complexes are not removed from the circulation as rapidly in SLE since the reticuloendothelial system may be 'blocked' due to immune complexes which are a feature of SLE. Another possibility is that platelet-leukocyte complexes arise as a consequence of auto-immune disease, or as part of an acute phase reaction. For this reason, patients with another 'chronic' autoimmune disorder (RA) were also studied. Only platelet-leukocyte complexes were analysed in these patients, hence one cannot exclude the presence of platelet degranulation, however it is unlikely to have been any more signficant than that found in SLE patients without secondary APS. The median values of platelet-granulocyte and platelet-monocyte complexes were significantly

higher in RA patients compared to controls, suggesting that platelet-leukocyte complexes are increased in auto-immune diseases, however it is possible that a higher median platelet count in RA may be partly responsible for this finding.

It has been shown that 'unactivated' platelets (i.e. those which have not undergone significant α-granule release as determined by CD62p expression) are capable of binding to isolated neutrophil and monocyte fractions at relatively low numbers of platelet per leukocyte (Rinder *et al*, 1991b). Significant percentages of neutrophils and monocytes bind 'unactivated' platelets in a quantitatively different manner, and the kinetics of platelet adhesion to monocytes and neutrophils is such that monocyte adhesion is favoured over neutrophil adhesion. The role of leukocyte stimulation in the formation of platelet-leukocyte complexes is somewhat unclear. One group have found that selective activation of leukocytes by N-formyl-methionyl - leucyl-phenylalanine (fMLP), resulted in no increase in the percentage of activated leukocyte-resting platelet conjugates for any leukocyte subset (Rinder *et al*, 1994). This appears to contrast directly with other reports. A recent paper has shown that fMLP alone increases platelet-leukocyte aggregates dose-dependently in unfixed whole blood (Li *et al*, 1997). One group have reported that fMLP-induced neutrophil activation 'switches-on' adhesive mechanisms contributing to neutrophil-platelet interaction alone or together with P-selectin (Evangelista *et al*, 1995). Data obtained with monoclonal antibodies suggests that CD18 (a leukocyte β2 integrin) is involved in this event. It has also been reported that resting platelets do not adhere to resting neutrophils, but markedly adhered to fMLP-activated neutrophils, although the number of platelets carried by neutrophils was five to six times lower with respect to activated platelets (Evangelista *et al*, 1996). This paper concludes that platelet-

neutrophil adhesion in dynamic conditions can be modelled as a multistep cascade - an initial P-selectin-dependent recognition step, followed by the neutrophil β2 integrin CD11b/CD18 activation, which in turn, through a subsequent adhesion step, stabilises platelet/neutrophil interaction. Platelet-monocyte adhesion is also thought to involve interactions which are independent of P-selectin, such as that between TSP and CD36 (Silverstein *et al*, 1989). Thus, it is likely that multiple mechanisms are responsible for the formation of platelet-leukocyte aggregates and that several receptors are involved. The relative importance of each may vary according to the stimulus responsible for the initial platelet and/or leukocyte activation.

In Chapter 3, patients receiving aspirin therapy had significantly lower median values for CD63 expression than those who were not on aspirin. This finding was not reproduced in the current study however. Reasons for the discrepancy include differences in patient selection between the two studies, as well as the small numbers of patients investigated.

Within both PAPS and SLE patient groups, there was a significantly positive correlation between only a few of the platelet activation markers, however this was not a consistent finding. A positive correlation existed between all three types of leukocyte-complexes in PAPS and SLE as would be expected. This lack of correlation between platelet activation markers is not an unexpected finding, since they do not necessarily reflect the same process. Some agonists are more likely to result in degranulation and changes in the conformational structure of GP IIb/IIIa rather than aminophospholipid exposure and microparticle formation. Platelet-leukocyte formation probably involves several mechanisms apart from platelet

activation as discussed previously. Finally, it is possible that the specific therapy each patient was receiving may effect various aspects of platelet activation.

Plasma levels of soluble P-selectin were significantly elevated in both PAPS and SLE patients compared to controls, and were significantly higher in PAPS than in SLE. Since soluble P-selectin is a marker of platelet (and probably endothelial cell) activation, this is consistent with the previous findings of increased platelet activation primarily in PAPS. Another possibility for the difference between P-selectin levels in PAPS and SLE is the use of immunosuppressive therapy in SLE patients. In PAPS, there was only a weak positive correlation between levels of soluble P-selectin and platelet CD63 expression. The significance of these findings has been discussed at length in Chapter 3 and will not be reproduced here.

Activity of the procoagulant pathway was also examined by measuring levels of TAT, F 1+2 and D-dimers. Since many of the patients were receiving oral anticoagulant therapy at the time of the study, all results should be interpreted with this in mind. Although median TAT and F 1+2 levels were similar between controls, PAPS and SLE patients, some individual in both PAPS and SLE patient groups had elevated levels of these markers, and the highest TAT level was found in an SLE patient without secondary APS. Median TAT levels were significantly higher in non-warfarinised patients compared to controls, and also compared to patients on warfarin. On the other hand, median F 1+2 levels were significantly reduced in warfarinised patients compared to controls and those not on warfarin, however, there was no significant difference in median F 1+2 levels between non warfarinised patients and controls. Although median levels of D-dimers were not significantly different in PAPS or SLE patients compared to controls, there were several patients with elevated

levels of D-dimers. All of these findings are similar to those found in Chapter 3, and the significance of increased thrombin generation in PAPS has been discussed previously. Increased thrombin generation has also been described in patients with SLE. One study reported elevated levels of TAT in SLE patients without APA compared to controls, and also compared to patients with APA (Yamazaki *et al*, 1994). It suggested that levels of TAT were higher in the SLE group because of other processes in SLE contributing to a further rise in TAT levels (eg hepatic or renal dysfunction).

Similar to the findings reported in Chapter 3, there was a significant negative correlation between INR and both TAT and F 1+2 levels. All patients receiving oral anticoagulants had normal/reduced levels of TAT and F 1+2, unlike the case in Chapter 3 where a patient with 'adequate' anticoagulation had an elevated level of TAT. The significance of the relationship between INR and markers of thrombin generation has been discussed at length in Chapter 3.

Endothelial cell activation markers (soluble E-selectin and TM) were significantly increased in both PAPS and SLE patients compared to controls. In the previous study, differences in soluble E-selectin levels between PAPS patients and controls failed to reach significance, but this may have been due to differences in patient selection. There was also a positive correlation between serum creatinine and TM levels in both groups of patients, and serum creatinine and soluble E-selectin levels in PAPS patients. Thus it is important to measure creatinine levels when performing these assays in order to correctly interpret the results. TM has been shown to be a sensitive marker of organ involvement and vasculitis in patients with SLE, not related to the presence of APA (Ohdama *et al*, 1994). Previous studies of plasma TM

levels in SLE patients have demonstrated a correlation with active lupus nephritis (Takaya *et al*, 1991), and extent of SLE clinical activity (Boheme *et al*, 1994), however all patients in the current study appeared to have stable, chronic disease.

An analysis of all assayed parameters demonstrated a number of significant correlations between platelet activation, thrombin generation and endothelial activation, particularly in SLE patients. It is difficult to understand the significance of these findings, since many of the parameters are likely to have been variably affected by the therapy that the patient was receiving, however it was interesting to note the positive relationship between platelet-leukocyte complexes and thrombin generation in patients with SLE. One possible explanation for this finding may be that formation of platelet-leukocyte complexes upregulates monocyte tissue factor expression, which in turn may affect the activity of the procoagulant pathway.

A number of differences were noted between SLE patients with APS compared to SLE patients without APS. In particular, SLE patients with APS had a significantly lower platelet count than those without APS. Although thrombocytopaenia forms part of the ARA diagnostic criteria for SLE, it is possible that the coexistence of APS may in some way exacerbate any existing thrombocytopaenia, however it it thought that APA themselves are not responsible for the reduced platelet count seen in APS (Galli *et al*, 1996a). Another possible explanation for this finding may be that some of the SLE patients were incorrectly classified as having APS - five of the fourteen patients classified as having SLE with secondary APS had thrombocytopaenia as their only clinical manifestation of APS, however, all of these patients had antibodies to β_2GPI, and several of them had positive LA and/or high titres of IgG/IgM ACL so it is difficult to exclude APS.

There was no significant difference in median values for any of the platelet activation markers CD62p, CD63, annexin V and PAC-1 between the two groups of SLE patients. SLE patients without APS had significantly higher levels of thrombin generation markers TAT and F 1+2 compared to SLE patients with APS. This is likely to be due to the fact that many of the patients with secondary APS were receiving oral anticoagulant therapy at the time of study. Another interesting finding was that SLE patients with APS had significantly higher TM levels than those patients without APS, buth this was probably related to the significantly higher levels of serum creatinine in the former group.

Screening for the FVL mutation and the prothrombn gene G20210A mutation (PGM) detected two patients (one with PAPS, one with SLE) who were heterozygote for the FVL mutation, whilst none of the fifty patients studied had the PGM detected. The PAPS patient with FVL had a history of arterial thrombosis only, and did not have a more severe form of thrombotic disease compared with the other patients. The SLE patient with FVL had no thormbotic history, was APA negative, but did have a history of one miscarriage. These findings are similar to those from chapter 3, and the significance of these prevalences has been discussed in detail previously.

In conclusion, the observations made in this chapter, largely support the findings of increased platelet activation, thrombin generation and endothelial cell activation in PAPS patients that was demonstrated in Chapter 3. As well as this, a more extensive investigation of platelet activation revealed significantly increased levels of PAC-1 binding, and in some cases, increased levels of circulating platelet-leukocyte complexes in PAPS. The fact that the levels of other platelet activation markers (i.e. annexin V binding and numbers of microparticles) were not significantly

elevated, indicates that the type of platelet activation in PAPS is limited, and does not appear to involve increased levels of platelet aminophospholipid exposure (although this cannot be completely excluded). Aspirin did not appear to have a signficant effect on expression of the platelet activation markers studied, however patient numbers were small. Patients with SLE (both with and without APS) also appear to have evidence of increased endothelial cell activation, as well as thrombin generation (except for those patients on warfarin). Despite having little evidence of increased platelet activation, SLE patients had the highest levels of circulating platelet-leukocyte complexes, and this may be related to factors such as leukocyte or even endothelial cell activation, as well as reduced clearance of these complexes from the circulation. Many have postulated that APA (and possibly other autoantibodies) play a causative role in the pathogenesis of APS, and so these antibodies may be responsible for many of the above findings. Numerous animal models imply that APA are pathogenic, but whether this is so in humans remains to be resolved. In the final part of this thesis, the possible platelet activating role of APA will be determined in a series of *in vitro* experiments, whereby the expression of platelet degranulation markers will be measured following an incubation with purified IgG from PAPS patients.

5. Investigation of the *in vitro* effects of purified APS IgG on platelet activation

5.1 Introduction

In the previous two chapters, evidence of *in vivo* platelet activation, thrombin generation and endothelial cell activation were demonstrated in patients with APS. Many investigators have proposed that APA are responsible for these observed changes, and play a causative role in the pathogenesis of thrombosis. Indeed, a mouse model for thrombosis has demonstrated the thrombogenic potential of IgG from patients with APS (Pierangeli *et al*, 1994a). Although these findings may not necessarily extrapolate to humans, they do support the notion that APA are pathogenic. It is likely that thrombosis in APS is probably a 'two-hit' phenomenon (Roubey 1998). Autoantibodies (the 'first-hit') are continually present in the circulation, however a local trigger ('second-hit') is required in order to induce thrombosis at a particular site in the vasculature at a particular time. Thus autoantibodies may cause a prothrombotic state in which thrombosis is triggered by local stimuli that would not normally be sufficient to do so (see section 1.3.1). One model which has been suggested for the pathogenesis of thrombosis in APS has parallels with the thrombosis of heparin-induced thrombocytopaenia (HIT) (Arnout 1996). It proposes that as a consequence of an initial damage, activation results in local exposure of negatively charged phospholipids on the surface of platelets, endothelial cells or on trophoblasts. These potentially reactive phospholipids are covered by phospholipid binding proteins such as β_2GPI or prothrombin. If APA are present and directed against such surface bound proteins, they will concentrate on the

cell surface, bind to cellular FcγRII receptors, and induce thrombosis-promoting changes. In some studies, APA have been shown to bind to platelets, however it appears that platelet activation and/or damage is a prerequisite for antibody binding to occur (Khamashta *et al*, 1988; Mikhail *et al*, 1988; Shi *et al*, 1993).

Many studies investigating the *in vitro* effects of APA positive plasma/serum or affinity purified IgG on normal platelets have been published. In some cases, there appears to be no effect on enhancing platelet activation/aggregation (Ichikawa *et al*, 1990; Out *et al*, 1991; Ford *et al*, 1998), whereas other investigators have reported a stimulatory effect of APA on platelets which have been previously 'primed' by a low dose of agonist (Robbins *et al*, 1998; Campbell *et al*, 1995; Nojima *et al*, 1999). In this current chapter, IgG was purified from patients with well documented PAPS, and a series of *in vitro* experiments involving incubation with normal platelets were performed in order to investigate a possible platelet-activating action of APA. Flow cytometric measurement of degranulation was used to detect platelet activation. The details of the methods used and the results obtained form the basis of this final results chapter.

5.2 Methods

5.2.1 Patients and controls

The study group consisted of six patients (five females and one male) with well documented PAPS who had been studied in the previous chapters. All patients had a history of thrombosis, and four of the five females had a history of recurrent miscarriage. All patients had a positive LA test and elevated ACL IgG titres. Five patients had antibodies to β_2GPI.

Normal plasma (for IgG purification) was collected from six healthy controls, and pooled prior to IgG purification. Normal platelet rich plasma for flow cytometry studies was obtained from three healthy donors who were not taking any medication at the time of the study.

5.2.2 Immunoglobulin G isolation

Reagents

All reagents were from Merck Ltd unless otherwise stated.

- Hi-Trap Protein G Sepharose (Pharmacia Biotech Ltd) packed in a 1 × 12 cm column
- Phosphate buffered saline, pH 7.2 (PBS) - 0.01 M phosphate, 0.145 M sodium chloride
- Glycine buffer - 0.1 M glycine, pH 2.8
- 1.0 M Tris, pH 9.0

Method

Plasma samples from six PAPS patients and the pooled control sample were diluted 1 in 5 in PBS and then applied to the Protein G Sepharose column which had been pre-equilibrated with PBS. After washing the column, bound protein was eluted with 0.1 M glycine buffer and collected in 4 ml fractions into tubes containing 0.4 ml of 1 M Tris. The protein was then dialysed to PBS and concentrated using an Amicon concentrator and PM30 membrane (Amicon Ltd). Protein concentration was determined by measuring the absorbance at 280 nm with a 1 cm light path and extinction coefficient $E^{1\%,\ 1cm} = 13.6$ (Johnstone & Thorpe 1987) and the protein was then aliquoted and stored at -70°C. Purity of the samples was determined by SDS-

PAGE under non-reducing conditions using the PhastSystem (Pharmacia Biotech) and gels demonstrated a single band corresponding to IgG.

5.2.3 Preparation of platelet rich plasma

Platelet rich plasma (PRP) was prepared from citrated anticoagulated blood collected from three healthy controls. Samples were centrifuged at 180 *g* for 10 minutes at room temperature. PRP was removed and stored at room temperature in a capped plastic tube. PPP (obtained by centrifuging samples at 2000 *g* for 15 minutes) or buffer (see below) was then added to the sample to adjust the platelet count to 250 × 10^9/l.

5.2.4 *In vitro* incubation of PRP with purified IgG

Reagents

- Monoclonal antibodies anti-CD62p-PE and anti-CD63-FITC (Immunotech, Beckman Coulter)
- Isotype controls IgG1-PE and IgG1-FITC (Immunotech, Beckman Coulter)
- Annexin V Fluos (Boehringer Mannheim) - made up in 10 µl aliquots and stored at -20°C
- HEPES buffered saline (HBS) - NaCL 0.145 mol/l, KCl 5 mmol/l, $MgSO_4$ 1 mmol/l, HEPES 10 mmol/l, pH 7.4
- K_2EDTA-HBS - 5 mmol/l K_2EDTA made up in HEPES buffered saline
- Ca-HBS - 2.5 mmol/l $CaCL_2$ made up in HEPES buffered saline
- PBS
- Tyrodes buffer - NaCL 0.137 mol/l, KCl 2.7 mmol/l, $NaH_2.PO_4.2H_20$ 0.4 mmol/l, $MgCl_2.6H_2O$ 2.0 mmol/l, $NaHCO_3$ 12 mmol/l, pH 6.5, with calcium 2.5 mmol/l

- Formaldehyde solution - made up to 0.2% in 0.145 mol/l NaCL, and filtered with a Sartorius Minisart 0.2 μm syringe filter (Fisher Scientific) prior to use. A fresh aliquot was made each day.
- Platelet agonists - thrombin related activation peptide (TRAP) (Sigma-Aldrich), ADP (Sigma-Aldrich)

Methods

20 μl PRP (platelet count adjusted to 250 × 10^9/l in PPP) was incubated with 25 μl pooled normal or patient IgG (at a final concentration of 2.5 mg/ml) or HBS (control) in a polypropylene microcentrifuge eppendorf tube for 30 minutes at room temperature. Following this, 5 μl aliquots of the sample were then incubated together with 5 μl monoclonal CD62-PE/isotype control and 40 μl HBS (with or without ADP at a final concentration of 1 μM) for a further 20 minutes at room temperature, before being fixed with 0.5 ml of 0.2% formalin saline. Samples were then analysed on the flow cytometer according to the protocol outlined in section 2.2.3, and antibody binding was expressed as the percentage of platelets staining positive for CD62-PE. A total of 10,000 events per sample were analysed.

In a separate series of *in vitro* experiments, a number of alterations were made to the above method in order to examine the effects of several variables on platelet activation. These included:

1. performing the initial incubation at 37°C instead of at room temperature
2. using monoclonal antibody CD63-FITC or annexin V-FITC to detect platelet activation instead of CD62-PE (in the experiments where annexin V-FITC was used, HBS and formalin saline fixative were substituted with the buffers K_2EDTA-HBS or Ca-HBS as outlined in section 2.2.5)

3. adding the agonist ADP to PRP together with IgG in the inital incubation step
4. varying the concentrations of IgG (final IgG concentration ranging from 313 μg/ml to 5 mg/ml)
5. diluting PRP into a calcium containing Tyrode's buffer instead of PPP
6. using 1.0/1.5 μM TRAP instead of 1 μM ADP as the platelet agonist

5.3 Results

Platelet CD62p was measured in PRP samples incubated with HBS alone, pooled normal IgG or patient IgG both with and without 1 μM ADP. Results obtained from using three normal platelet donors are shown below in *Table 5.1*.

PRP incubated with:	% CD62p expression Donor 1 PRP		% CD62p expression Donor 2 PRP		% CD62p expression Donor 3 PRP	
	No ADP	1 μM ADP	No ADP	1 μM ADP	No ADP	1 μM ADP
HBS	3.2	26.9	6.6	17.6	2.0	12.2
Normal IgG	3.5	21.5	6.5	20.5	2.3	17.4
Patient 1 IgG	3.1	24.0	2.9	21.6	1.5	18.1
Patient 2 IgG	3.1	27.9	3.0	22.3	1.9	16.2
Patient 3 IgG	2.5	26.7	3.9	19.8	2.0	15.1
Patient 4 IgG	2.8	31.3	1.9	16.7	2.0	16.5
Patient 5 IgG	4.1	25.9	3.5	20.6	1.6	13.7
Patient 6 IgG	2.7	24.5	3.0	18.4	1.3	10.7

Table 5.1 *% Platelet CD62p expression following PRP incubation with HBS, normal IgG, or six different PAPS patient IgG's (with and without ADP).*

In all donors, the addition of 1 μM ADP resulted in a significant increase in platelet CD62p expression (as expected). In both unstimulated and ADP stimulated samples of PRP, the addition of normal or patient IgG resulted in either no change, a slight increase, or in some cases a slight decrease in CD62p expression. None of the

patient IgG's tested had a consistent inhibitory or stimulatory effect on platelet CD62p expression for all three donors, and similarly no significant difference was found between normal IgG and any of the patient IgG's for all three donors. Although the incubation of donor 1 PRP wih patient 4 IgG and ADP appeared to result in a significant increase in CD62p expression (when compared with normal IgG), this finding was not replicated when the same patient IgG was incubated with other donor PRP.

The failure to detect a platelet activating effect of APS IgG using the previously described experimental conditions resulted in a series of other experiments being performed, whereby a number of variables were altered. Representative results from some of these experiments are summarised in *Table 5.2.* None of these changes in methodology (i.e.performing the incubation at 37°C, altering the platelet agonist, measuring CD63 expression or annexin V binding, using Tyrodes-calcium buffer, using a higher concentration of IgG, or incubating PRP and agonist together) resulted in any significant differences in the results obtained between normal IgG and patient IgG. In some experiments performed at 37°C, the incubation of stimulated PRP with normal or patient IgG resulted in a significant decrease in platelet CD62p and CD63 expression compared with incubation of PRP with buffer alone. This was not due to an obvious pH or ionic strength effect.

Experimental Conditions	Unstimulated PRP			Stimulated PRP		
	No IgG	Normal IgG	Patient IgG	No IgG	Normal IgG	Patient IgG
Incubation at 37°C Platelet agonist 1 μM TRAP **% CD62p measured**	1.8	2.2	1.9	60.6	26.5	28.0
Incubation at 37° Platelet agonist 1 μM TRAP **% CD63 measured**	3.1	3.9	4.8	25.2	12.4	14.5
Incubation at RT Platelet agonist 1μM ADP **% CD63 measured**	2.1	3.5	2.0	3.9	4.7	3.6
Incubation at RT No platelet agonist **% Annexin V binding measured**	3.6	2.5	2.4	-	-	-
PRP diluted in Tyrodes-calcium buffer Incubation at RT Platelet agonist 1 μM ADP **%CD62p measured**	3.2	4.2	4.8	45.9	37.9	34.5
IgG final concentration 5 mg/ml Incubation at RT Platelet agonist 1 μM ADP **% CD62p measured**	3.2	3.5	4.2	26.9	21.5	22.9
PRP, IgG and agonist (1.5 μM TRAP) incubated together **% CD63 measured**	1.6	2.5	1.5	17.7	17.4	16.6

Table 5.2 *Platelet activation (%CD62p, CD63 or annexin V-binding) in in vitro experiments performed under varying conditions*

5.4 Discussion

Many investigators have suggested that APA are pathogenic and play an important role in thrombosis, which is a feature of APS. The thrombogenic potential of APS IgG has been demonstrated in mice subjected to a standardised 'pinch' injury (Pierangeli *et al*, 1994a). In this model, mice passively immunised with IgG-APS prior to the 'pinch', developed a significantly larger thrombus than those immunised with saline, and the thrombus persisted longer in a significantly higher number of mice immunised with IgG-APS compared to mice immunised with either saline or IgG-normal. Such a finding has led to the consideration that the same thrombogenic potential may exist in humans. The concept of antibody-mediated thrombosis is not a new one, and there are similarities between the thrombosis of APS and HIT (Arnout 1996). That APA may result in platelet activation (under suitable conditions) is supported by the findings that APA can bind to platelets. Affinity purified ACA have been demonstrated to bind to immobilised platelets bound to a solid phase (Hasselaar *et al*, 1990), however it may be that such binding only occurs following platelet injury or PS exposure.

In this chapter, the potential platelet-activating abilities of APS IgG were investigated in a series of *in vitro* experiments utilising normal PRP. Even though it is recognised that steps such as centrifugation (used to prepare PRP) can result in platelet activation, for these set of *in vitro* experiments it was not considered to be problematic, since a low level of activation was considered optimal and subthreshold doses of platelet agonists were also used. Platelet activation was initially assessed by measuring percentage CD62p expression following incubation of normal PRP (from three different donors) with purified IgG, and no consistent difference was found

between normal IgG and patient IgG. Since it is possible that APA may only bind to platelets following their activation or injury, the *in vitro* experiments were repeated with the addition of subthreshold doses of ADP. However, there was still no consistent difference found in CD62p expression between PRP incubated with normal IgG compared to patient IgG. Although some patient IgG's appeared to result in an increase in CD62p expression following ADP stimluation of a particular donor PRP, these findings were not replicated with the other donor PRP, and are probably within the CV's for the assay. In some cases, the addition of IgG resulted in a reduction in measured platelet activation, but again, this finding was not replicated with all platelet donors. As a result of these negative findings, alterations were made to the basic methodology to determine if variations in the experimental conditions may result in different findings. Similar results were demonstrated, and in some experiments performed at 37°C, incubation of stimulated PRP with normal or patient IgG resulted in a significant reduction in platelet CD62p and CD63 expression, compared with incubation of PRP with buffer alone. This was not due to a pH or ionic strength effect and is difficult to explain. Thus it was concluded that under the conditions of these *in vitro* experiments, APS IgG do not induce platelet activation.

The findings from previously published studies examining the *in vitro* effects of APA on platelet activation/aggregation are inconsistent. It has been shown that the incubation of thrombin activated platelets with ACL/β_2GPI complexes from patients with APS, results in a significantly increased *in vitro* TXB_2 production (Robbins *et al*, 1998). The Fab fragments from LA and ACL positive patients IgG have been shown to increase platelet aggregation and ^{14}C serotonin release of platelets stimulated by low dose thrombin (Martinuzzo *et al*, 1993). Plasma from SLE patients with APA

can promote platelet aggregation when investigated under flow conditions in a perfusion system, whereas SLE plasmas without APA had no such effect (Escolar *et al*, 1991). Several flow cytometric studies of *in vitro* platelet activation have also been performed, and one group compared the effects of affinity purified IgG ACL antibodies from patients with APS and syphilis on platelet CD62p expression and aggregation (Campbell *et al*, 1995). They found that in the presence of low concentration thrombin, ADP or collagen, all IgG-APS induced platelet aggregation and activation, but none of the IgG-syphilis had this effect. In the absence of agonists, only 50% IgG-APS caused platelet aggregation and none caused platelet activation. A recently published paper has also examined the *in vitro* effects of ACL and LA on the enhancement of platelet CD62p expression (Nojima *et al*, 1999). They reported that platelet CD62p was significantly augmented by ACL+LA+ plasma/purified IgG from ACL+LA+ plasma in combination with low concentrations of ADP. However, this did not occur with samples that were ACL–LA+, ACL+ LA–, or ACL–LA–. This is in direct contrast with the findings of the current study in which all six APS IgG (purified from patients who were both LA+ and ACL IgG+) had no effect on platelet CD62p expression, even in the presence of ADP. One possible explanation for this discrepancy is that alternative procedural methods were used in the other study, and this may be partly responsible for the different results. Another consideration is the possibility that normal donor PRP has a variable reactivity to APA. Certainly it has been recognised that some donor platelets are more 'responsive' than others in their ability to aggregate in the presence of heparin-induced antibodies, however some of the donors used had been shown to respond to HIT sera on previous occasions. Finally, it is possible that polymorphisms of the platelet FcγRII phenotype may affect

the ability of APA to activate platelets. It is known that the Arg/Arg 131 phenotype binds human IgG_2 and IgG_3 only weakly compared with the His/His 131 phenotype (Rascu *et al*, 1997) and hence this may also be an important variable to consider.

On the other hand, numerous investigators have found no evidence of enhanced platelet activation/aggregation as a result of incubation with APA samples. Both LA positive and LA negative plasmas were found to equally enhance aggregation of normal platelets when stimulated with low concentrations of ADP (Ichikawa *et al*, 1990). One group reported that despite the specific binding of both LA Ig and ACL Ig to platelets (in the presence of thrombin ± β_2GPI), neither of these antibodies had an effect on thrombin-induced release of serotonin or βTG, nor did they affect platelet aggregation induced by a number of agonists (Shi *et al*, 1993). Similar findings have been published by other investigators. In one study, APA binding to circulating platelets was not associated with measurable aggregation abnormalities nor with increased platelet expression of CD63 (Out *et al*, 1991). Another group have more recently reported that despite the presence of platelet membrane binding IgG in approximately 50% of the APS patient sera tested, there was no evidence for increased platelet-activating ability as determined by platelet microparticle formation and P-selectin expression (Ford *et al*, 1998).

In conclusion, the results of this chapter have failed to demonstrate an *in vitro* platelet activating action of APA under the experimental conditions described. Nonetheless, evidence of *in vivo* platelet activation in APS patients was found in Chapters 3 and 4. Thus it is possible to conclude that APA themselves are not responsible for the platelet activation that was previously found in patients with PAPS. It is difficult however to explain the inconsistent results obtained from the

numerous *in vitro* studies cited above. It may be that the experimental conditions used in this chapter were not optimal for detecting platelet activation induced by APA. The concentration of IgG tested was comparable to that of other studies, hence it is unlikely that too low a concentration of IgG was used. One consideration is that PS exposure may be an important factor required for APA induced platelet activation, however it is difficult to induce PS exposure without causing maximal platelet degranulation in the *in vitro* setting. It is also possible that other cellular factors not present in normal PRP may be required for APA to induce platelet activation.

6. Summary and conclusions

The primary aim of this thesis was to examine *in vivo* platelet activation in patients with PAPS using flow cytometric methods. Some aspects of endothelial cell activation and thrombin generation were also assessed. The initial findings supported the theory that platelet activation was a feature of APS, hence a more extensive investigation of patients with PAPS and SLE was performed Some novel methods were also used to detect platelet activation, and development of more suitable methodology formed a significant part of the thesis work. Finally, an attempt was made to investigate the possibility that APA may have the ability to directly activate platelets.

Two major groups of patients were selected for study. The first group were PAPS patients with well documented disease, regularly attending a routine haemostasis clinic. Due to the pattern of patient referral, the majority of patients had a history of thrombotic disease. Many of these patients were receiving antiplatelet and anticoagulant therapy which could not be discontinued for the study. The second major group of patients studied were those with SLE, approximately half of whom also had secondary APS. These patients were attending a routine rheumatology clinic and also had well documented disease. The majority of SLE patients were on immunosuppressive treatment, and several patients were also receiving antiplatelet and anticoagulant therapy. A small subgroup of patients with RA were selectively studied for purposes of providing a 'control' autoimmune group for one of the assays (platelet-leukocyte complexes). In particular, all of the above patients had stable, chronic disease and were not recovering from a recent thrombotic epsiode.

Much of the previous work performed on platelet activation utilised methods which were either insensitive or prone to artefactual changes. The advent of whole blood flow cytometry to detect platelet activation was a major step, since it was not necessary to prepare PRP (which can lead to *in vitro* activation), and it was more sensitive than the older methods. However it is important to note that flow cytometry is still affected by pre-test variables (such as tramuatic venepuncture or sampling from non-resting patients), and so specific procedural steps were still required to ensure that artefactual platelet activation did not occur. Previous reports had suggested that platelet activation may play an important part in the thrombosis of APS, and many studies examining platelet activity/aggregation in this disorder had been published. However, there had been a limited number of studies performed using flow cytometric tecniques, hence it was felt that this topic could be investigated further. Since platelets do not act in isolation in haemostasis, activity of the procoagulant pathway as well as the endothelium were also examined.

In the initial investigation (Chapter 3), twenty PAPS patients were studied. Platelet activation and turnover, thrombin generation, and endothelial cell activation were assessed. Median levels of CD63 expression were increased in PAPS patients compared to a control group, and were also sigificantly higher in those patients not on aspirin (although patient numbers were small). Median levels of CD62p expression were similar between patients and controls, however soluble P-selectin levels were significantly increased in patients. This was not an unexpected finding, as it has been shown that circulating degranulated platelets rapidly lose their surface P-selectin to the plasma pool. There was no correlation between platelet CD63 expression and soluble P-selectin, which may have been partly due to the fact that activated

endothelial cells may also be a source of soluble P-selectin, and that their half-lives may differ. There was no correlation between markers of platelet activation and the presence or absence of LA, ACL or antibodies to β_2GPI. Reticulated platelet percentages were similar in patients and controls, and patients with mild thrombocytopaenia did not have elevated levels, suggesting that platelet turnover was not increased. In most patients, PFA-100™ analysis did not reveal a shortening of closure times, and prolonged closure times secondary to the defect induced by aspirin therapy were demonstrated .

Changes in markers of thrombin generation were observed in the patient group. Median TAT levels were significantly higher in non-warfarinised patients compared to those on warfarin and controls. On the other hand, median F 1+2 levels were significantly reduced in warfarinised patients compared to controls and those patients not on warfarin. This suggested that there is increased thrombin generation occurring in PAPS, and that anticoagulants appeared to have a greater effect on F 1+2 reduction than on TAT. An interesting finding was that one patient on adequate anticoagulant therapy (as determined by INR and chromogenic factor VII and X assays) had an elevated TAT level (and normal F1+2 level), which suggested that thrombin generation may still occur despite therapy with warfarin. Median levels of D-dimer and fibrinogen were not significantly elevated in the patient group.

There was some evidence of increased endothelial cell activation in the PAPS group. Median values of plasma TM were signficantly higher in PAPS patients compared to contols, however there was a significant correlation between the levels of TM and serum creatinine (as has been previously described), despite the fact that most patients had normal renal function. Differences in E-selectin levels between patients

and controls did not reach statistical significance, however there were some individual patients with high E-selectin values. There was no correlation between levels of E-selectin and creatinine, which suggest that it may be a more useful marker of endothelial injury in the presence of renal impairment. There was no correlation between E-selectin and TM levels in PAPS patients, and this is probably due the effect of renal dysfunction on TM levels, but it may also be because the release of these two soluble markers from the endothelium is under the control of different factors.

Only one patient was found to be heterozygous for the Factor V Leiden mutation, and the prothrombin gene G20210A variant was not detected in any of the patients. These findings are consistent with those from several other population studies. The one patient with FVL mutation did not have a more significant thrombotic history than those patients without this inherited defect.

The findings from Chapter 3 supported the hypothesis that *in vivo* platelet activation occurred in PAPS, and there was also evidence of both endothelial activation and thrombin generation. Due to the effects of therapy, it was not possible to determine whether or not there was a significant correlation between markers of platelet or endothelial activation and those of thrombin generation. Whilst this initial work was being conducted, a number of new flow cytometric methods which allow the detection of other aspects of platelet activation were published. These included the detection of platelet aminophospholipid exposure (using annexin V binding), circulating platelet-leukocyte complexes, and numbers of platelet microparticles in platelet poor plasma. Hence it was felt that a more extensive investigation of platelet activation in PAPS using these techniques was appropriate. The methodology was

developed, and a further group of PAPS patients were studied. SLE patients were also investigated, since secondary APS is not uncommon in this condition, and they provided a 'control' autoimmune group for comparison with PAPS. Many of the assays already performed in Chapter 3 were repeated in Chapter 4, along with the newly developed methods.

In this second study, many of the results from the previous chapter were confirmed. Median CD63 expression in PAPS patients was increased compared to controls, as were plasma levels of soluble P-selectin. Interestingly, median CD63 expression was signficantly higher in PAPS patients compared to those SLE patients with secondary APS. One possible explanation for this finding was that the majority of SLE patients were receiving immunosuppressive therapy and this may in some way affect platelet activation. Another marker of platelet activation (PAC-1 binding) was significantly increased in PAPS patients and SLE patients with secondary APS, providing further evidence of *in vivo* platelet activation in this condition. Median levels of annexin V binding and numbers of platelet microparticles were similar amongst the groups studied, however several patients had increased levels of both markers. One possible reason for normal levels in most patients is that significant platelet PS exposure and microparticle formation is unlikely to occur under normal resting conditions, since *in vitro* experiments have shown that strong agonists or mechanical disruption of platelets are usually required for maximal expression. Alternatively, it is also posssible that highly reactive PS expressing platelets and microparticles are rapidlly cleared from the circulation. Platelet-leukocyte complexes were generally increased in SLE and in some cases, PAPS patients. Median levels of all three types of platelet-leukocyte complexes were significantly higher in SLE

compared to PAPS, which suggested that factors other than platelet activation were also responsible for their formation since platelet activation was found more frequently in PAPS than in SLE patients. Indeed a small group of patients with RA were also found to have elevated levels of platelet-leukocyte complexes compared to controls. It is also possible that platelet-leukocyte complexes are not removed as quickly from the circulation in SLE or RA compared to PAPS. Since these diseases are systemic in nature, is is possible that extensive immune complex formation may block the reticuloendothelial system and interfere with the removal of platelet-leukocyte complexes.

Thrombin generation was increased in some patients from both the PAPS and SLE groups, and the results were similar to those obtained in Chapter 3. Median TAT levels were significantly higher in non-warfarinised patients compared to those on warfarin and controls, whislt median F 1+2 levels were significantly reduced in warfarinised patients compared to controls and those patients not on warfarin. Interestingly, SLE patients without secondary APS had significantly higher levels of TAT complexes than normal controls (but not PAPS patients), and this suggests that other processes in SLE (such as systemic inflammation, and hepatic or renal dysfunction) may contribute to a further rise in TAT. Median levels of D-dimers were not significantly higher in PAPS or SLE patients, although some individuals in both groups had increased values.

Evidence of endothelial cell activation was found in PAPS and SLE patients, with increased median levels of E-selectin and TM found in both groups. As with the findings in Chapter 3, there was a significant correlation between TM levels and

serum creatinine. TM is also known to be a sensitive marker of organ involvement and vasculitis in patients with SLE, not related to the presence of APA.

Within the group of patients with SLE, there were some significant differences between those with APS and those without secondary APS. In particular, SLE patients with APS had a significantly lower median platelet count, and twelve of the fourteen patients with secondary APS had antibodies to β_2GPI, compared to none in the non-APS group. This suggests that the presence of antibodies to β_2GPI in SLE patients is highly indicative of secondary APS, and may have a role in future testing.

Only two patients were found to be heterozygous for the Factor V Leiden mutation, and the prothrombin gene G20210A variant was not detected in any of the patients. One PAPS patient with FVL had a history of arterial thrombosis only, and did not have a more severe form of thrombotic disease compared with the other patients. One SLE patient with FVL had no thormbotic history, was APA negative, but did have a history of one miscarriage. Thus there did not appear to be an clustering of these genetic defects in patients with thrombotic APS.

The findings from Chapters 3 and 4 supported the theory that *in vivo* platelet activation occurred in PAPS. From these results, it seemed reasonable to hypothesise that APA may have platelet activating effects, particularly since the role of heparin-induced antibodies in the thrombosis of HIT was well recognised. In Chapter 5, an investigation of the *in vitro* effects of purified IgG from six APS patients on normal platelets was performed using both stimulated and unstimulated PRP from normal donors. Under the experimental conditions, no evidence of a platelet-enhancing effect of APA was found. This was in agreement with the results from some studies, but in direct contrast with the results from other groups. Despite some alterations to the basic

methods used, it was still not possible to detect platelet activation in the presence of APS IgG. This led to the conclusion that either APA themselves did not cause platelet activation, or alternatively, that the experimental conditions used were not optimal for detecting platelet activation induced by APA.

A large number of patients that were studied had a significant thrombotic history and therefore required long term antiplatelet and/or anticoagulant therapy which could not be discontinued. In the initial study, patients receiving aspirin appeared to have lower median levels of platelet CD63 expression than those not on aspirin, however this finding was not duplicated in the next study. This discrepancy may have occurred because of the small numbers of patients studied, however it is also known that platelet activation can occur in spite of aspirin therapy. These findings may suggest that aspirin is not always the most effective antiplatelet therapy in this condition, and that newer antiplatelet agents may have a role to play in the prophylaxis of arterial thrombosis in APS. In most cases, warfarin was effective in reducing thrombin generation, although the effect was most significant on F1+2 levels rather than TAT levels. Indeed, one warfarinised patient had a significantly elevated level of TAT (despite a 'therapeutic' INR), and this suggests that in some patients, assaying TAT and F1+2 levels may be useful, particularly when they have a thrombotic event whilst on anticoagulants. Since the results of the Warfarin in AntiPhospholipid Syndrome (WAPS) study are awaited, the optimal level of anticoagulation in APS is still debated, and hence measuring markers of thrombin generation may be helpful in guiding therapy in some cases.

In summary, the work performed in this thesis provided evidence of *in vivo* platelet activation, thrombin generation and endothelial cell activation in PAPS.

Some of these findings were reproduced in SLE patients, both with and without secondary APS, thus it is not entirely clear whether APA were the causative factor, simply an epiphenomen, or possibly markers of another process. Nevertheless, many of the techniques used to detect platelet activation were recently developed and thus gave some insight into the nature of platelet activation in these disorders. Clearly, the events that occur upon platelet activation are not an 'all or nothing' phenomenon, and studying platelet activation using flow cytometry allows one to detect various aspects of the activation process (e.g. degranulation, PS exposure, or microparticle formation). In particular, this may have important implications for future antiplatelet therapies. Although a platelet-activating effect of APA was not demonstrated in this thesis, I would like to continue further work in this area as I believe that there is a strong link between platelets and APA, based on the model of antibody-induced thrombosis which occurs in HIT. Finally, I would also like to study the platelet FcγRII polymorphism in APS patients, since it may affect the ability of a patient's platelets to be activated by APA.

7. References

Amengual O, Atsumi T, Khamashta, M.A. and Hughes GRV (1998) The role of tissue factor pathway in the hypercoagulable state in patients with the antiphospholipid syndrome. *Thromb Haemost* **79**, 276-281.

Ames PR, Pyke S, Iannaccone, L. and Brancaccio, V. (1995) Antiphospholipid antibodies, haemostatic variables and thrombosis - a survey of 144 patients. *Thromb Haemost* **73**, 768-773.

Ames, P.R., Tommasino, C., Iannaccone, L., Brillante, M., Cimino, R. and Brancaccio, V. (1996) Coagulation activation and fibrinolytic imbalance in subjects with idiopathic antiphospholipid antibodies--a crucial role for acquired free protein S deficiency. *Thromb.Haemost.* **76**, 190-194.

Ames PR, Tommasino, C., D'Andrea G, Iannaccone, L., Brancaccio, V. and Margaglione M (1998) Thrombophilic genotypes in subjects with idiopathic antiphospholipid antibodies - prevalence and significance. *Thromb Haemost* **79**, 46-49.

Andree HAM, Hermens WT, Hemker HC and Willems GM (1992) Displacement of factor Va by annexin V. In: Andree HAM, (Ed.) *Phospholipid binding and anticoagulant action of annexin V*, pp. 73-85. Maastricht, the Netherlands: Universitaire Pers Maastricht

Angles-Cano E, Sultan Y and Clauvel JP (1979) Predisposing factors to thrombosis in systemic lupus erythematosus. *J Lab Clin Med* **94**, 312-323.

Arnout J (1996) The pathogenesis of the antiphospholipid syndrome: a hypothesis based on parallelisms with heparin-induced thrombocytopenia. *Thromb Haemost* **75**, 536-541.

Aruffo A, Kolanus W, Walz G, Fredman P and Seed B (1991) CD62/P-selectin recognition of myeloid and tumor cell sulfatides. *Cell* **67**, 35-44.

Arvieux, J., Roussel, B., Pouzol, P. and Colomb, M.G. (1993) Platelet activating properties of murine monoclonal antibodies to beta 2-glycoprotein I. *Thromb.Haemost.* **70**, 336-341.

Asherson RA, Khamashta, M., Ordi-Ros J, Derksen RHWM, Machin SJ, Barquinero J, Outt HH, Harris EN, Vilardell-Torres M and Hughes GRV (1989) The "primary" antiphospholipid syndrome: major clincial and serological features. *Medicine (Baltimore)* **68**, 366-374.

Asherson RA (1992) The catastrophic antiphospholipid syndrome. *J Rheumatol* **19**, 508-512.

Asherson RA (1998) The catastrophic antiphospholipid syndrome, 1998. A review of the clinical features, possible pathogenesis and treatment. *Lupus* **7**, S55-S62

Atsumi T, Khamashta, M.A., Ames PRJ, Ichikawa K, Koike T and Hughes GRV (1996) Effect of β_2 glycoprotein I and human monoclonal anticardiolipin antibody on the protein S/C4b-binding protein system. *Lupus* **6**, 358-364.

Ault KA and Knowles C (1995) *In vivo* biotinylation demonstrates that reticulated platelets are the youngest platelets in the circulation. *Exp Hematol* **23**, 996-1001.

Bancsi LF, van der Linden IK and Bertina RM (1992) β_2 glycoprotein-I deficiency and the risk of thrombosis. *Thromb Haemost* **67**, 649-653.

Barcellona D, Biondo G, Mameli G and Marongiu F (1997) Are prothrombin fragment 1+2 and thrombin-antithrombin complexes useful in the management of oral anticoagulant therapy? *Int J Clin Lab Res* **27**, 76-78.

Berman CL, Yeo EL, Wencel-Drake J, Furie BC, Ginsberg MH and Furie B (1986) A platelet alpha-granule protein that is associated with the plasma membrane after activation: characterisation and subcellular localisation of platelet-activation dependent granule-external membrane protein. *J Clin Invest* **78**, 130-137.

Bevers EM, Comfurius P and Zwaal RFA (1983) Changes in membrane phospholipid distribution during platelet activation. *Biochim Biophys Acta* **736**, 57-66.

Bevers EM, Galli M, Barbui, T., Comfurius P and Zwaal RFA (1991) Lupus anticoagulant IgGs (LA) are not directed to phospholipids only, but to a complex of lipid-bound prothrombin. *Thromb Haemost* **66**, 629-632.

Biasiolo, A. and Pengo, V. (1993) Antiphospholipid antibodies are not present in the membrane of gel- filtered platelets of patients with IgG anticardiolipin antibodies, lupus anticoagulant and thrombosis. *Blood Coagul.Fibrinolysis* **4**, 425-428.

Bihour, C., Durrieu-Jais, C., Besse, P., Nurden, P. and Nurden, A.T. (1995) Flow cytometry reveals activated GP IIb-IIIa complexes on platelets from patients undergoing thrombolytic therapy after acute myocardial infarction. *Blood Coagul.Fibrinolysis* **6**, 395-410.

Boey ML, Loizou S, Colaco CB, Matkin JA and Hughes GRV (1984) Antithrombin III in systemic lupus erythematosus. *Clin.Exp.Rheumatol.* **2**, 53-56.

Boffa M-C and Karmochkine M (1998) Thrombomodulin: an overview and potential implications in vascular disorders. *Lupus* **7**, S120-S125

Boheme MWJ, Nawroth PP, Kling E, Lin J, Amiral J, Riedesel J, Raeth U and Scherbaum WA (1994) Serum thrombomodulin. A novel marker of disease activity in systemic lupus erythematosus. *Arthritis Rheum* **4**, 572-577.

Bowie EJW, Thompson Jr, J., Pascuzzi CA and Owen Jr, C. (1963) Thrombosis in systemic lupus erythematosus despite circulating anticoagulants. *J Lab Clin Med* **62**, 416-430.

Bowry SK, Prentice CRM and Courtney JM (1985) A modification of the Wu and Hoak method for the determination of platelet aggregates and platelet adhesion. *Thromb Haemost* **53**, 381-385.

Branch DW, Dudley DJ, Mitchell MD, Creighton KA, Abbott TM, Hammond EH and Daynes RA (1990) Immunoglobulin G fractions from patients with antiphospholipid antibodies cause fetal death in BALB/c mice: a model for autoimmune fetal loss. *Am J Obstet Gynecol* **163**, 210-216.

Brey RL and Escalante A (1998) Neurological manifestations of antiphospholipid antibody syndrome. *Lupus* **7**, S67-S74

Brighton, T.C., Hogg PJ, Dai Y-P, Murray BH, Chong, B.H. and Chesterman, C.N. (1996) Beta$_2$-glycoprotein I in thrombosis: evidence for a role as a natural anticoagulant. *Br J Haematol* **93**, 185-194.

Broze Jr GJ and Gailani d (1993) The role of factor XI in coagulation. *Thromb Haemost* **70**, 72-74.

Broze Jr GJ (1995) Tissue factor pathway inhibitor and the current concept of blood coagulation. *Blood Coagul Fibrinolysis* **6**, S7-S13

Burstein SA and Breton Gorius J (1995) Megakaryopoiesis and platelet formation. In: Beutler E et al (Eds.) *Williams Hematology*, 5th edn. pp. 1149-1160. New York: McGraw Hill

Campbell, A.L., Pierangeli, S.S., Wellhausen, S. and Harris, E.N. (1995) Comparison of the effects of anticardiolipin antibodies from patients with the antiphospholipid syndrome and with syphilis on platelet activation and aggregation. *Thromb.Haemost.* **73**, 529-534.

Cariou R, Tobelem G, Belluci S, Soria J, Soria C, Maclouf J, Caen J (1988) Effect of lupus anticoagulant on antithrombogenic properties of endothelial cells - inhibition of thrombomodulin-dependent protein C activation. *Thromb Haemost* **60**, 54-58.

Carreras, L.O. and Vermylen JG (1982) 'Lupus' anticoagulant and thrombosis - possible role of inhibition of prostacyclin formation. *Thromb Haemost* **48**, 38-40.

Celi A, Furie B and Furie BC (1991) PADGEM: an adhesion receptor for leukocytes on stimulated platelets and endothelial cells. *Proc Soc Exp Biol Med* **198**, 703-709.

Ceriello A, Pirisi M, Giacomello R, Stel G, Faletti E, Motz E, Lizzio S, Gonano F and Bartoli E (1994) Fibrinogen plasma levels as a marker of thrombin activation: new insights on the role of fibrinogen as a cardiovascular risk factor. *Thromb Haemost* **71**, 595

Chong, B.H., Murray B, Berndt, M.C., Dunlop, L.C., Brighton, T.C. and Chesterman, C.N. (1994) Plasma P-selectin is increased in thrombotic consumptive platelet disorders. *Blood* **83**, 1535-1541.

Chong, B.H., Brighton, T.C. and Chesterman, C.N. (1995) Antiphospholipid antibodies and platelets. *Semin.Thromb.Hemost.* **21**, 76-84.

Chow TW, Hellums JD, Moake JL and Kroll MH (1992) Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. *Blood* **80**, 113-120.

Chronos, N.A., Wilson, D.J., Janes, S.L., Hutton, R.A., Buller, N.P. and Goodall, A.H. (1994) Aspirin does not affect the flow cytometric detection of fibrinogen binding to, or release of alpha-granules or lysosomes from, human platelets. *Clin.Sci.(Colch.)* **87**, 575-580.

Colucci M, Balconi G, Lorenzet R, Locati D, Donati MB and Semerano N (1983) Cultured human endothelial cells generate tissue factor in response to endotoxin. *J Clin Invest* **71**, 1893-1896.

Combes V, Dignat-George F, Mutin M and Sampol J (1997) A new flow cytometry method of platelet-derived microvesicle quantitation in plasma. *Thromb Haemost* **77**, 220

Conley CL and Hartmann RC (1952) A hemorrhagic disorder caused by circulating anticoagulant in patients with disseminated lupus erythematosus. *J Clin Invest* **31**, 621-622.

Cuadrado MJ, López-Pedrera C, Khamashta, M.A., Camps MT, Tinahones F, Torres A, Hughes GRV and Velasco F (1997) Thrombosis in primary antiphospholipid syndrome. A pivotal role for monocyte tissue factor expression. *Arthritis Rheum* **40**, 834-841.

Dahlback B, Nilsson IM and Frohm B (1983) Inhibition of prothrombinase activity by a lupus anticoagulant. *Blood* **62**, 218-223.

Dale GL, Friese P, Hynes LA and Burstein SA (1995) Demonstration that thiazole orange-positive platelets in the dog are less than twenty-four hours old. *Blood* **85**, 1-4.

Davie EW and Ratnoff OD (1964) Waterfall sequence for intrinsic blood clotting. *Science* **145**, 1310-1312.

Daviet L and McGregor JL (1997) Vascular biology of CD36: roles of this new adhesion molecule family in different disease states. *Thromb Haemost* **78**, 65-69.

de Bruijne-Admiraal LG, Modderman PW, Von dem Borne AEGKr and Sonnenberg A (1992) P-selectin mediates Ca^{2+}-dependent adhesion of activated platelets to many different types of leukocytes: detection by flow cytometry. *Blood* **80** , 134-142.

de Groot, P.G., Oosting, J.D. and Derksen, R.H. (1993) Antiphospholipid antibodies: specificity and pathophysiology. *Baillieres.Clin.Haematol.* **6**, 691-709.

Della Valle P, Crippa L, Safa O, Tomassini L, Pattarini E, Vigano-D'angelo S, Grazia Sabbadini M and D'Angelo A (1996) Potential failure of the International Normailzed Ration (INR) system in the monitoring of oral anticoagulation in patients with lupus anticoagulants. *Ann Med Interne (Paris)* **147,** Suppl 1, 10-14.

Diacovo TG, Roth SJ, Buccola KM, Bainton DF and Springer TA (1996) Neutrophil rolling, arrest, and transmigration across activated, surface adherent platelets via sequential action of P-selectin and the β_2-integrin CD11b/CD18. *Blood* **88**, 146-157.

Diez-Ewald, M., Torres-Guerra, E., Vizcaino, G. and Arteaga-Vizcaino, M. (1995) Platelet function in patients with lupus anticoagulant and thrombosis. *Invest.Clin.* **36**, 13-21.

Dunlop, L.C., Skinner, M.P., Bendall, L.J., Favaloro, E.J., Castaldi, P.A., Gorman, J.J., Gamble, J.R., Vadas, M.A. and Berndt, M.C. (1992) Characterization of GMP-140 (P-selectin) as a circulating plasma protein. *J.Exp.Med.* **175**, 1147-1150.

Escolar, G., Font, J., Reverter, J.C., Lopez-Soto, A., Garrido, M., Cervera, R., Ingelmo, M., Castillo, R. and Ordinas, A. (1992) Plasma from systemic lupus erythematosus patients with antiphospholipid antibodies promotes platelet aggregation. Studies in a perfusion system. *Arterioscler.Thromb.* **12**, 196-200.

Evangelista V, Manarini S, Martelli N, de Gaetano G, McGregor JL and Cerletti C (1995) fMLP increases platelet (Plt) adhesion to granulocuytes (Grc): role of CD18. *Thromb Haemost* **73**, 1084 (Abstract)

Evangelista V, Manarini S, Rotondo S, Martelli N, Polischuk R, McGregor JL, de Gaetano G and Cerletti C (1996) Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the β_2 integrin CD11b/CD18. *Blood* **88**, 4183-4194.

Falanga A, Ofosu FA, Cortelazzo S, Delaini F, Marziali S and Barbui, T. (1994) Hemostatic system activation in patients with lupus anticoagulant and essential thrombocytemia. *Semin.Thromb.Hemost.* **20**, 324-327.

Fanelli, A., Bergamini, C., Rapi, S., Caldini, A., Spinelli, A., Buggiani, A. and Emmi, L. (1997) Flow cytometric detection of circulating activated platelets in primary antiphospholipid syndrome. Correlation with thrombocytopenia and anticardiolipin antibodies. *Lupus.* **6**, 261-267.

Feinstein DI and Rapaport SI (1972) Acquired inhibitors of blood coagulation. *Prog Hemost Thromb* **1**, 75-95.

Fijnheer, R., Frijns, C.J., Korteweg, J., Rommes, H., Peters, J.H., Sixma, J.J. and Nieuwenhuis, H.K. (1997) The origin of P-selectin as a circulating plasma protein. *Thromb.Haemost.* **77**, 1081-1085.

Finazzi, G., Barbui, T. and for the Provisional Steering Committee of the WAPS Study (1996) Feasibility of a randomized clinical trial for the prevention of recurrent thrombosis in the antiphospholipid syndrome: the WAPS project. *Ann Med Interne (Paris)* **147**, 38-41.(Abstract)

Firkin BJ, Howard MA and Radford N (1980) Possible relationship between lupus inhibitor and recurrent abortions in young women. *Lancet* **ii**, 366

Forastiero, R., Kordich LC, Basilotta E and Carreras LO (1994) Differences in protein S and C4b-binding protein in different groups of patients with antiphospholipid antibodies. *Blood Coagul Fibrinolysis* **37**, 1453-1461.(Abstract)

Forastiero RR, Martinuzzo MM, Cerrato GS, Kordich LC and Carreras LO (1997) Relationship of antiβ_2-glycoprotein I and antiprothrombin antibodies to thrombosis and pregnancy loss in patients with antiphospholipid antibodies. *Thromb Haemost* **78**, 1008-1014.

Forastiero, R., Martinuzzo, M., Carreras, L.O. and Maclouf, J. (1998) Anti-beta2 glycoprotein I antibodies and platelet activation in patients with antiphospholipid antibodies: association with increased excretion of platelet-derived thromboxane urinary metabolites. *Thromb.Haemost.* **79**, 42-45.

Ford, I., Urbaniak, S. and Greaves, M. (1998) IgG from patients with antiphospholipid syndrome binds to platelets without induction of platelet activation. *Br.J.Haematol.* **102**, 841-849.

Foxall C, Watson SR, Dowbenko D, Fennie C, Lasky LA, Kiso M, Hasegawa A, Asa D and Bradley BK (1992) The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis X oligosaccharide. *J Cell Biol* **117**, 895-902.

Francis Jr RB and Neely S (1989) Effect of lupus anticoagulant on endothelial fibrinolytic activity *in vitro*. *Thromb Haemost* **61**, 314-317.

Fukudome K and Esmon CT (1994) Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem* **269**, 26486-26491.

Furie B and Furie BC (1997) Leukocyte crosstalk at the vascular wall. *Thromb Haemost* **78**, 306-309.

Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, Hechtman HB and Michelson AD (1998) Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J Am Coll Cardiol* **31**, 352-358.

Galli M, Comfurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJ, Barbui, T., Zwaal RFA and Bevers EM (1990) Anticardiolipin antibodies (ACA) are directed not to cardiolipin but to a plasma protein cofactor. *Lancet* **335**, 1544-1547.

Galli, M., Barbui, T., Comfurius P, Zwaal RFA and Bevers EM (1992) Anticoagulant activity of anticardiolipin antibodies is mediated by β2-glycoprotein I. *Thrombosis and Haemostasis* **68**, 297-300.

Galli M, Grassi A and Barbui, T. (1993) Platelet-derived microvesicles in patients with antiphospholipid antibodies. *Thromb Haemost* **69**, 541(Abstract)

Galli, M., Finazzi, G. and Barbui, T. (1996a) Thrombocytopenia in the antiphospholipid syndrome. *Br J Haematol* **93**, 1-5.

Galli, M., Grassi, A. and Barbui, T. (1996b) Platelet-derived microvesicles in thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *Thromb.Haemost.* **75**, 427-431.

Galli M and Barbui, T. (1999) Antiprothrombin antibodies: detection and clinical significance in the antiphospholipid syndrome. *Blood* **93**, 2149-2157.

Gawaz, M.P., Mujais, S.K., Schmidt, B. and Gurland, H.J. (1994) Platelet-leukocyte aggregation during hemodialysis. *Kidney Int.* **46**, 489-495.

Geng JG, Bevilacqua MP, Moore KL, McIntyre TM, Prescott SM, Kim JM, Bliss GA, Zimmerman GA and McEver RP (1990) Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature* **343**, 757-760.

George JN and Dale GL (1995) Platelet kinetics. In: Beutler E et al (Eds.) *Williams Hematology*, 5th edn. pp. 1202-1205. New York: McGraw Hill

Gewirtz AM and Schick B (1994) Megakaryocytopoiesis. In: Colman RW, Hirsh J, Marder VJ and Salzman EW, (Eds.) *Hemostasis and thrombosis: basic principles and clinical practice*, Third edn. pp. 353-396. Philadelphia: J B Lippincott

Gharavi, A., Pierangeli, S.S., Gharavi EE., Hua T, Barker JH, Anderson G and Harris EN (1998) Thrombogenic properties of antiphospholipid antibodies do not depend on their binding to β_2 glycoprotein I (β2 GPI) alone. *Lupus* **7**, 341-346.

Glas-Greenwalt P, Kant KS, Allen C and Pollak VE (1984) Fibrinolysis in health and disease: severe abnormalities in systemic lupus erythematosus. *J Lab Clin Med* **104**, 962-976.

Godeau B, Piette J-C, Fromont P, Intrator L, Schaeffer A and Bierling P (1997) Specific antiplatelet glycoprotein autoantibodies are associated with the thrombocytopaenia of primary antiphospholipid syndrome. *Br J Haematol* **98**, 873-879.

Gschwandtner ME, Eichinger S, Hütter D, Korninger L, Lechner K, Panzer S and Pabinger I (1996) Lupus anticoagulant and thromboembolism: evaluation of fibrinogen, natural inhibitors and molecular markers of thrombosis. *Blood Coagul Fibrinolysis* **7**, 325-330.

Haga, H.J., Christopoulos, C., Machin, S.J., Khamashta, M. and Hughes, G.R. (1992) Lack of specific binding of anticardiolipin antibodies to intact platelets. *Lupus.* **1**, 387-390.

Halbmayer W-M, Haushofer A, Schön R and Fischer M (1994) Influence of lupus anticoagulant on a commercially available kit for APC-resistance. *Thromb Haemost* **72**, 645-646.

Hamburger SA and McEver RP (1990) GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood* **75**, 550-554.

Harris, E.N., Gharavi, A.E., Boey ML, Patel BM, Mackworth-Young CG, Loizou S, Hughes GRV (1983) Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet* **2**, 1211-1214.

Harris, E.N. (1987) Syndrome of the black swan. *Br J Rheumatol* **26**, 324-326.

Hasselaar P, Derksen RHWM, Blokzijl L, Hessing M, Nieuwenhuis, H.K., Bouma BN and de Groot PG (1989) Risk factors for thrombosis in lupus patients. *Ann.Rheum.Dis.* **48**, 933-940.

Hasselaar P, Derksen RHWM, Blokzijl L and deGroot PG (1990) Crossreactivity of antibodies directed against cardiolipin, DNA, endothelial cells and blood platelets. *Thromb Haemost* **63**, 169-173.

Hasselaar, P., Derksen, R.H., Blokzijl, L. and de Groot, P.G. (1988) Thrombosis associated with antiphospholipid antibodies cannot be explained by effects on endothelial and platelet prostanoid synthesis. *Thromb.Haemost.* **59**, 80-85.

Holmsen H, Kaplan KL and Dangelmaier CA (1982) Differential energy requirements for platelet responses: A simultaneous study of aggregation, three secretory processes, arachidonate liberation, phosphatidylinositol turnover and phosphatidate production. *Biochem J* **208**, 9-18.

Horstman LL, Jy W, Schultz DR, Mao WW and Ahn YS (1994) Complement-mediated fragmentation and lysis of opsonized platelets: gender differences in sensitivity. *J Lab Clin Med* **123**, 515-525.

Hughes GRV (1983) Thrombosis, abortion, cerebral disease and lupus anticoagulant. *Br Med J* **187**, 1088-1089.

Hughes GRV (1984) Connective tissue disease and the skin. *Clin Exp Dermatol* **9**, 535-544.

Hunt JE, McNeil HP, Morgan GJ, Creighton KA and Krilis SA (1992) A phospholipid-β2-glycoprotein I complex is an antigen for anticardiolipin antibodies occurring in autoimmune disease but not with infection. *Lupus* **1**, 75-81.

Hutton RA, Laffan MA and Tuddenham EGD (1998) Normal haemostasis. In: A Victor Hoffbrand, S Mitchell Lewis and Edward GD Tuddenham, (Eds.) *Postgraduate Haematology*, 4th edn. pp. 550-580. Oxford: Butterworth Heinemann

Ichikawa, Y., Kobayashi, N., Kawada, T., Shimizu, H., Moriuchi, J., Ono, H., Watanabe, K. and Arimori, S. (1990) Reactivities of antiphospholipid antibodies to blood cells and their effects on platelet aggregations in vitro. *Clin.Exp.Rheumatol.* **8**, 461-468.

Israels SJ, Gerrard JM, Jacques YV, McNicol A, Cham B, Nishibori M and Bainton DF (1992) Platelet dense granule membranes contain both granulophysin and P-selectin (GMP-140). *Blood* **80**, 143-152.

Johnstone A and Thorpe R (1987) *Immunochemistry in practice*, Oxford: Blackwell Scientific Publications.

Jones DW, Gallimore MJ, Harris SL and Winter M (1999) Antibodies to factor XII associated with lupus anticoagulant. *Thromb Haemost* **81**, 387-390.

Jungi TW, Spycher MO, Nydegger UE and Barandun S (1986) Platelet leukocyte interaction: selective binding of thrombin-stimulated platelets to human monocytes, polymorphonuclear leukocytes, and related cell lines. *Blood* **67**, 629-636.

Jy, W., Horstman, L.L., Arce, M. and Ahn, Y.S. (1992) Clinical significance of platelet microparticles in autoimmune thrombocytopenias. *J.Lab.Clin.Med.* **119**, 334-345.

Kaplan KL (1994) Laboratory markers of platelet activation. In: Colman RW, Hirsh J, Marder VJ and Salzman EW, (Eds.) *Hemostasis and thrombosis: basic principles and clinical practice*, Third edn. pp. 1180-1196. Philadelphia: J B Lippincott

Keeling DM, Campbell SJ, Mackie IJ, Machin SJ and Isenberg DA (1991) The fibrinolytic response to venous occlusion and the natural anticoagulants in patients with antiphospholipid antibodies both with and without systemic lupus erythematosus. *Br J Haematol* **77**, 354-359.

Keeling DM, Wilson AJG, Mackie IJ, Machin SJ and Isenberg DA (1992) Some antiphospholipid antibodies bind to β_2-glycoprotein I in the absence of phospholipid. *Br J Haematol* **82**, 571-574.

Keeling, D.M. and Isenberg, D.A. (1993) Haematological manifestations of systemic lupus erythematosus. *Blood Rev.* **7**, 199-207.

Khamashta, M.A., Harris, E.N., Gharavi, A.E., Derue, G., Gil, A., Vazquez, J.J. and Hughes, G.R. (1988) Immune mediated mechanism for thrombosis: antiphospholipid antibody binding to platelet membranes. *Ann.Rheum.Dis.* **47**, 849-854.

Khamashta, M.A., Cuadrado MJ, Mujic F, Taub NA, Hunt BJ and Hughes GRV (1995) The management of thrombosis in the antiphospholipid-antibody syndrome. *N Engl J Med* **332**, 993-997.

Khamashta, M.A. (1998) Management of thrombosis and pregnancy loss in the antiphospholipid syndrome. *Lupus* **7**, S162-S165

Kieffer N, Guichard N, Farcet JP, Vainchenker W and Breton Gorius J (1987) Biosynthesis of major platelet proteins in human platelets. *Eur J Biochem* **164**, 189-195.

Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti R, Wagner DD and Furie B (1989) PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* **59**, 305-312.

Larsen E, Palabrica T, Sajer SA, Gilbert GE, Wagner DD, Furie BC and Furie B (1990) PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). *Cell* **63**, 467-474.

Lawrie AS, Purdy G, Mackie IJ and Machin SJ (1997) Monitoring of oral anticoagulant therapy in lupus anticoagulant positive patients with the anti-phospholipid syndrome. *Br J Haematol* **98**, 887-892.

Lechner K and Pabinger-Fasching (1985) Lupus anticoagulant and thrombosis. *Hemostasis* **15**, 254-262.

Lee, D.H., Warkentin, T.E., Denomme, G.A., Hayward, C.P. and Kelton, J.G. (1996) A diagnostic test for heparin-induced thrombocytopenia: detection of platelet microparticles using flow cytometry. *Br.J.Haematol.* **95**, 724-731.

Lellouche, F., Martinuzzo, M., Said, P., Maclouf, J. and Carreras, L.O. (1991) Imbalance of thromboxane/prostacyclin biosynthesis in patients with lupus anticoagulant. *Blood* **78**, 2894-2899.

Levy RA, Avvad E, Oliveira J and Porto LC (1998) Placental pathology in antiphospholipid syndrome. *Lupus* **7**, S81-S85

Levy Y, Ziporen L, Gilburd B, George J, Polak-Charcon S, Amital H, Cledes J, Youimou P and Shoenfeld Y (1996) Membranous nephropathy in primary antiphospholipid syndrome: description of a case and induction of renal injury in SCID mice. *Hum Antibodies Hybridoma* **7**, 91-96.

Li N, Goodall AH and Hjemdahl P (1997) A sensitive flow cytometric assay for circulating platelet-leucocyte aggregates. *Br J Haematol* **99**, 808-816.

Lo SC, Salem HH, Howard MA, Oldmeadow MJ and Firkin BG (1990) Studies of natural anticoagulant proteins and anticardiolipin antibodies in patients with the lupus anticoagulant. *Br J Haematol* **76**, 380-386.

Lorant DE, Topham MK, Whatley RA, McEver RP, McIntyre TM, Prescott SM and Zimmerman GA (1993) Inflammatory roles of P-selectin. *J Clin Invest* **92**, 559-570.

Lorant DE, McEver RP, McIntyre TM, Moore KL, Prescott SM and Zimmerman GA (1995) Activation of PMNLs reduces their adhesion to P-selectin and causes redistribution of ligands for P-selectin on their surfaces. *J Clin Invest* **96**, 171-182.

MacFarlane RG (1964) An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature* **202**, 498-499.

Malia RG, Kitchen S, Greaves, M. and Preston FE (1990) Inhibition of activated protein C and its cofactor protein S by antiphospholipid antibodies. *Br J Haematol* **79**, 101-107.

Mannucci PM, Bottasso B, Tripodi A and Bianchi Bonomi A (1991) Prothrombin fragment 1+2 and intensity of treatment with oral anticoagulants. *Thromb Haemost* **66**, 741

Marciniak E and Romond EH (1989) Impaired catalytic function of activated protein C : a new *in vitro* manifestation of lupus anticoagulant. *Blood* **74**, 2426-2432.

Martinuzzo, M.E., Maclouf, J., Carreras, L.O. and Levy-Toledano, S. (1993) Antiphospholipid antibodies enhance thrombin-induced platelet activation and thromboxane formation. *Thromb.Haemost.* **70**, 667-671.

Martinuzzo MM, Forastiero RR and Carreras LO (1995) Anti β_2 glycoprotein I antibodies: detection and association with thrombosis. *Br J Haematol* **89**, 397-402.

Matsuura E, Igarashi Y, Fujimoto M, Ichikawa K, Koike T (1990) Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. *Lancet* **336**, 177-178.

Matsuura E, Igarashi Y, Yasuda T, Triplett DA and Koike T (1994) Anticardiolipin antibodies recognise β_2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. *J Exp Med* **179**, 457-462.

McEver RP (1991) Leukocyte interactions mediated by selectins. *Thromb Haemost* **66**, 80-87.

McNally T, Mackie IJ, Machin SJ and Isenberg DA (1995a) Increased levels of β_2 glycoprotein-I antigen and β2 glycoprotein-I binding antibodies are associated with a history of thromboembolic complications in patients with SLE and primary antiphospholipid syndrome. *Br J Rheumatol* **34**, 1036

McNally T, Mackie IJ, Machin SJ and Isenberg DA (1995b) Elevated levels of β_2 glycoprotein-I (β2GPI) in antiphospholipid antibody syndrome are due to increased amounts of β2GPI in association with other plasma constituents. *Blood Coagul Fibrinolysis* **6**, 411-416.

McNeil HP, Chesterman, C.N. and Krilis, S.A. (1989) Anticardiolipin antibodies and lupus anticoagulants comprise separate antibody subgroups with different phospholipid binding characteristics. *Br.J.Haematol.* **73**, 506-513.

McNeil HP, Simpson RJ, Chesterman, C.N. and Krilis SA (1990) Antiphospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: β_2 glycoprotein I. *Proc Natl Acad Sci USA* **87**, 4120-4124.

McNeil HP, Chesterman, C.N. and Krilis SA (1991) Immunology and clinical importance of antiphospholipid antibodies. *Adv Immunol* **49**, 193-280.

McVerry BA, Machin, S.J., Parry H and Goldstone AH (1980) Reduced prostacyclin activity in systemic lupus erythematosus. *Ann.Rheum.Dis.* **39**, 524-525.

Metcalfe, P., Williamson, L.M., Reutelingsperger, C.P., Swann, I., Ouwehand, W.H. and Goodall, A.H. (1997) Activation during preparation of therapeutic platelets affects deterioration during storage: a comparative flow cytometric study of different production methods. *Br.J.Haematol.* **98**, 86-95.

Michelson AD, Barnard MR, Hechtman HB, MacGregor H, Connolly RJ, Loscalzo J and Valeri CR (1996) *In vivo* tracking of platelets: circulating degranulated platelets rapidly lose their surface P-selectin but continue to circulate and function. *Proc Natl Acad Sci USA* **93**, 11877-11882.

Michelson, A.D. (1996) Flow cytometry: a clinical test of platelet function. *Blood* **87**, 4925-4936.

Mikhail MH, Szczech LAM and Shapiro SS (1988) The binding of lupus anticoagulants (LACs) to human platelets. *Blood* **72**, 333 (Abstract)

Minnema MC, Pajkrt D, Wuillemin WA, Roem D, Bleeker WK, Levi M, van Deventer SJH, Hack CE and ten Cate H (1998) Activation of clotting factor XI without detectable contact activation in experimental human endotoxaemia. *Blood* **92**, 3294-3301.

Miyamoto, S., Marcinkiewicz, C., Edmunds, L.H.J. and Niewiarowski, S. (1998) Measurement of platelet microparticles during cardiopulmonary bypass by means of captured ELISA for GPIIb/IIIa [In Process Citation]. *Thromb.Haemost.* **80**, 225-230.

Moake JL, Turner NA, Stathopoulos NA, Nolasco L, Hellums JD (1988) Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate and is resistant to aspirin. *Blood* 1366-1374.

Moll S and Ortel TL (1996) The INR is invalid in monitoring warfarin anticoagulant therpay in patients with lupus anticoagulants. *Lupus* **5**, 522

Montaruli B, Borchiellini A, Tamponi G, Giorda L, Bessone P, van Mourik JA, Voorberg J and Schinco P (1996) Factor V Arg506-Gln mutation in patients with antiphospholipid antibodies. *Lupus* **5**, 303-306.

Moore JE and Mohr CF (1952) Biologically false-positive serologic tests for syphilis: type, incidence, and cause. *JAMA* **150**, 467-473.

Moore JE and Lutz WB (1955) The natural history of systemic lupus erythematosus: an approach to its study through chronic biologic false positive reactions. *J Chronic Dis* **1**, 297-316.

Moore KL, Eaton SF, Lyons DE, Lichenstein HS, Cummings RD and McEver RP (1994) The P-selectin glycoprotein ligand from human neutrophils displays sialylated fucosylated, O-linked poly-N-acetyllactosamine. *J Biol Chem* **269**, 23318-23327.

Nawroth PP and Stern DM (1986) Modulation of endothelial cell haemostatic properties by tumour necrosis factor. *J Exp Med* **163**, 740

Nemerson Y (1988) Tissue factor and hemostasis. *Blood* **71**, 1-8.

Nieuwenhuis, H.K., van Oosterhout, J.J., Rozemuller, E., van Iwaarden, F. and Sixma, J.J. (1987) Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood* **70**, 838-845.

Nieuwland, R., Berckmans, R.J., Rotteveel-Eijkman, R.C., Maquelin, K.N., Roozendaal, K.J., Jansen, P.G., ten Have, K., Eijsman, L., Hack, C.E. and Sturk, A. (1997) Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. *Circulation* **96**, 3534-3541.

Nilsson IM, Astedt B, Hedner U and Berezin D (1975) Intrauterine death and circulating anticoagulant ("antithromboplastin"). *Acta Med Scand* **197**, 153-159.

Nimpf, J., Bevers, E.M., Bomans, P.H., Till, U., Wurm, H., Kostner, G.M. and Zwaal, R.F. (1986) Prothrombinase activity of human platelets is inhibited by beta 2-glycoprotein-I. *Biochim.Biophys.Acta* **884**, 142-149.

Nimpf, J., Wurm, H. and Kostner, G.M. (1987) Beta 2-glycoprotein-I (apo-H) inhibits the release reaction of human platelets during ADP-induced aggregation. *Atherosclerosis* **63**, 109-114.

Nishibori M, Cham B, McNicol A, Shalev A, Jain N and Gerrard JM (1993) The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky-Pudlak syndrome, and appears identical to granulophysin. *J Clin Invest* **91**, 1775-1782.

Nojima J, Suehisa E, Kuratsune H, Machii T, Koike T, Kitani T, Kanakura Y and Amino N (1999) Platelet activation induced by combined effects of anticardiolipin and lupus anticoagulant IgG antibodies in patients with systemic lupus erythematosus. *Thromb Haemost* **81**, 436-441.

Nurden AT and Nurden P (1993) A review of the role of platelet membrane glycoproteins in the platelet-vessel wall interaction. *Baillieres Clinical Haematol* **6**, 653-690.

Ohdama S, Yoshizawa Y, Kubota T and Aoki N (1994) Plasma thrombomodulin as an indicator of thromboembolic disease in systemic lupus erythematosus. *Int J Cardiol* **47**, 1-6.

Oosting, J.D., Derksen RHWM, Bobbink TWG, Hackeng TM, Bouma BN and de Groot PG (1993) Antiphospholipid antibodies directed against a combination of phospholipid with prothrombin, protein C, or protein S: an explanation for their pathogenic mechanism? *Blood* **81**, 2618-2625.

Out, H.J., de Groot, P.G., van Vliet, M., de Gast, G.C., Nieuwenhuis, H.K. and Derksen, R.H. (1991) Antibodies to platelets in patients with anti-phospholipid antibodies. *Blood* **77**, 2655-2659.

Owens, M.R. (1994) The role of platelet microparticles in hemostasis. *Transfus.Med.Rev.* **8**, 37-44.

Pablos JL, Caliz RA, Carreira PE, Atsumi T, Serrano L, Amengual O, Santiago B, Khamashta, M.A., Hughes GRV and Gomez-Reino JJ (1999) Risk of thrombosis in patients with antiphospholipid antibodies and factor V Leiden mutation. *J Rheumatol* **26**, 588-590.

Palabrica T, Lobb R, Furie BC, Aronovitz M, Benjamin C, Hsu Y-M, Sajer SA and Furie B (1992) Leukocyte accumulation promoting fibrin deposition is mediated *in vivo* by P-selectin on adherent platelets. *Nature* **359**, 848-851.

Palosuo T, Virtamo J, Haukka J, Taylor PR, Aho K, Puurunen M and Vaarala O (1997) High antibody levels to prothrombin imply a risk of deep venous thrombosis and pulmonary embolism in middle-aged men. A nested case-control study. *Thromb Haemost* **78**, 1178-1182.

Parke A, Weinstein RE, Bona RD, Maier DB and Walker FJ (1992) The thrombotic diathesis associated with the presence of phospholipid antibodies may be due to low level of free protein S. *Am J Med* **93**, 49-56.

Peters, M.J., Heyderman, R.S., Hatch, D.J. and Klein, N.J. (1997) Investigation of platelet-neutrophil interactions in whole blood by flow cytometry. *J.Immunol.Methods* **209**, 125-135.

Picillo U, De Lucia D, Palatiello E, Scuotto A, Marcialis MR, Pezzella S and Tirri G (1998) Association of primary antiphospholipid syndrome with inherited activated protein C resistance. *J Rheumatol* **25**, 1232-1234.

Picker LJ, Warnock RA, Burns AR, Doerschuk CM, Berg EL and Butcher EC (1991) The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell* **66**, 921-933.

Pierangeli, S.S., Barker JH, Stikovac D, Ackerman D, Anderson G, Barquinero J, Acland R and Harris EN (1994) Effect of human IgG antiphospholipid antibodies on an in vivo thrombosis model in mice. *Thromb Haemost* **71**, 670-674.

Pierangeli, S.S., Liu XW, Barker JH, Anderson G and Harris EN (1995) Induction of thrombosis in a mouse model by IgG, IgM and IgA immunoglobulins from patients with the antiphospholipid syndrome. *Thromb Haemost* **74**, 1367

Pierangeli, S.S., Liu XW, Anderson G, Barker JH and Harris EN (1996) Thrombogenic properties of murine anti-cardiolipin antibodies induced by β_2 glycoprotein I and human immunoglobulin G antiphospholipid antibodies. *Circulation* **94**, 1751

Piette J-C (1998) Towards improved criteria for the antiphospholipid syndrome. *Lupus* **7**, S149-S157

Polley MJ, Phillips ML, Wayner E, Nudelman E, Singhal AK, Hakomori S and Paulson JC (1991) CD62 and endothelial cells adhesion molecule-1 (ELAM-1) recognize the same carbohydrate ligand, sialyl Lewis x. *Proc Natl Acad Sci USA* **88**, 6224-6228.

Rai R, Cohen H, Dave M and Regan L (1997) Randomised controlled trial of aspirin and aspirin plus heparin in pregnant women with recurrent miscarriage associated with phospholipid antibodies. *BMJ* 253-257.

Rand JH, Wu X-X, Andree HAM, Lockwood CJ, Guller S, Scher J and Harpel, P.C. (1997) Pregnancy loss in the antiphospholipid-antibody syndrome - a possible thrombogenic mechanism. *N Engl J Med* **337**, 154-160.

Rand, J.H., Wu, X.X., Andree, H.A., Ross, J.B., Rusinova, E., Gascon-Lema, M.G., Calandri, C. and Harpel, P.C. (1998) Antiphospholipid antibodies accelerate plasma coagulation by inhibiting annexin-V binding to phospholipids: a "lupus procoagulant" phenomenon. *Blood* **92**, 1652-1660.

Rascu A, Repp R, Westerdaal NAC, Kalden JR and van de Winkel JGJ (2000) Clinical relevance of Fcγ receptor polymorphisms. *Ann NY Acad Sci* **815**, 282-295.

Rauch J (1998) Lupus anticoagulant antibodies: recognition of phospholipid-binding protein complexes. *Lupus* **7**, S29-S31

Redlich H, Vickers J, Kehrel B, Heptinstall S and Spangenberg P (1997) Formation of platelet-leukocyte conjugates in whole blood. *Platelets* **8**, 419-425.

Reverter, J.C., Tassies, D., Escolar, G., Font, J., Lopez, S.A., Ingelmo, M. and Ordinas, A. (1995) Effect of plasma from patients with primary antiphospholipid syndrome on platelet function in a collagen rich perfusion system. *Thromb.Haemost.* **73**, 132-137.

Rinder CS, Bonan JL, Rinder HM, Mathew J, Hines R and Smith BR (1992) Cardiopulmonary bypass induces leukocyte-platelet adhesion. *Blood* **79**, 1201-1205.

Rinder HM, Bonan JL, Rinder CS, Ault KA and Smith BR (1991) Dynamics of leukocyte-platelet adhesion in whole blood. *Blood* **78**, 1730-1737.

Rinder HM, Tracey JL, Rinder CS, Leitenberg D and Smith BR (1994) Neutrophil but not monocyte activation inhibits P-selectin mediated platelet adhesion. *Thromb Haemost* **71**, 750-756.

Rinder, H.M., Bonan, J.L., Rinder, C.S., Ault, K.A. and Smith, B.R. (1991) Activated and unactivated platelet adhesion to monocytes and neutrophils. *Blood* **78**, 1760-1769.

Robbins, D.L., Leung, S., Miller-Blair, D.J. and Ziboh, V. (1998) Effect of anticardiolipin/beta2-glycoprotein I complexes on production of thromboxane A2 by platelets from patients with the antiphospholipid syndrome. *J.Rheumatol.* **25**, 51-56.

Robert A, Le Querrec A, Delahousse B, Caron C, Houbouyan L, Boutière B, Horellou MH, Reber G and Sié for the Groupe "Méthodologie en Hémostase" du Groupe d'Etudes sur l'Hémostase et la Thrombose (1998) Control of oral anticoagulation in patients with the antiphospholipid syndrome - influence of the lupus anticoagulant on International Normalized Ratio. *Thromb Haemost* **80**, 99-103.

Robinson MSC, Mackie IJ, Khair K, Liesner R, Goodall AH, Savidge GF, Machin SJ and Harrison P (1998) Flow cytometric analysis of reticulated platelets: evidence for a large proportion of non-specific labelling of dense granules by fluorescent dyes. *Br J Haematol* **100**, 351-357.

Rosenberg RD and Bauer KA (1994) The heparin-antithrombin system: a natural anticoagulant mechanism. In: Colman RW, Hirsh J, Marder VJ and Salzman EW, (Eds.) *Hemostasis and thrombosis: basic principles and clinical practice*, Third edn. pp. 837-860. Philadelphia: JB Lippincott Company

Rosing J, van Rijn JLML, Bevers EM, van Dieijen G, Comfurius P and Zwaal RFA (1985) The role of activated human platelets in prothrombin and factor X activation. *Blood* **65**, 319-332.

Rosove MH, Brewer P and Runge A (1989) Simultaneous lupus anticoagulant and anticardiolipin assays and clinical detection of antiphospholipids. *Am.J.Hematol.* **32**, 148-149.

Roubey RAS, Pratt CW, Buyon JP and Winfield JB (1992) Lupus anticoagulant activity of autoimmune antiphospholipid antibodies is dependent β_2-glycoprotein I. *J Clin Invest* **90**, 1100-1104.

Roubey RAS, Eisenberg RA, Harper MF and Winfield JB (1995) 'Anti-cardiolipin' autoantibodies recognize β_2-glycoprotein I in the absence of phospholipid: importance of Ag density and bivalent binding. *J Immunol* **154**, 954-960.

Roubey RAS (1998) Mechanisms of autoantibody-mediated thrombosis. *Lupus* **7**, S114-S119

Ruf, A., Pick, M., Deutsch, V., Patscheke, H., Goldfarb, A., Rachmilewitz, E.A., Guillin, M.C. and Eldor, A. (1997) In-vivo platelet activation correlates with red cell anionic phospholipid exposure in patients with beta-thalassaemia major. *Br.J.Haematol.* **98**, 51-56.

Sako D, Chang X-J, Barone KM, Vachnio G, White HM, Shaw G, Veldman T, Bean KM, Ahern TJ, Furie B, Cumming DA and Larsen GR (1993) Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell* **75**, 1179-1186.

Salemink I, Blezer R, Willems GM, Galli M, Bevers EM and Lindhout T (1998) Antibodies to β_2-glycoprotein I from patients with antiphospholipid syndrome reduce the phospholipid-dependent inhibitory activity of tissue factor pathway inhibitor on factor X activation. *Lupus* **7**, S180(Abstract)

Schmaier AH (1997) Contact activation: a revision. *Thromb Haemost* **78**, 101-107.

Schmitz G, Rothe G, Ruf A, Barlage S, Tschöpe D, Goodall AH, Michelson AD, Nurden AT, Shankey TV and for the European working group on clinical cell analysis (1998) European working group on clinical cell analysis: consensus protocol for the flow cytometric characterisation of platelet function. *Thromb Haemost* **79**, 885-896.

Schousboe I (1985) Beta 2-Glycoprotein I: a plasma inhibitor of the contact activation of the intrinsic blood coagulation pathway. *Blood* **66**, 1086-1091.

Schultze HE, Heide K and Haput H (1961) Uber ein bisher ubekanntes niedermolekulars β2-globulin des humanserums. *Naturwissenschaften* **48**, 719-724.

Shattil, S.J., Hoxie, J.A., Cunningham, M. and Brass LF (1985) Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. *J Biol Chem* **260**, 11107-11114.

Shattil, S.J., Cunningham, M. and Hoxie, J.A. (1987) Detection of activated platelets in whole blood using activation- dependent monoclonal antibodies and flow cytometry. *Blood* **70**, 307-315.

Shechter Y, Tal Y, Greenberg A and Brenner B (1998) Platelet activation in patients with antiphospholipid syndrome. *Blood Coagul Fibrinolysis* **9**, 653-657.

Shi, W., Chong, B.H. and Chesterman, C.N. (1993) Beta 2-glycoprotein I is a requirement for anticardiolipin antibodies binding to activated platelets: differences with lupus anticoagulants. *Blood* **81**, 1255-1262.

Shibata, S., Harpel, P.C., Gharavi, A., Rand, J. and Fillit, H. (1994) Autoantibodies to heparin from patients with antiphospholipid antibody syndrome inhibit formation of antithrombin III-thrombin complexes [see comments]. *Blood* **83**, 2532-2540.

Silverstein RL and Nachman RL (1987) Thrombospondin binds to monocytes-macrophages and mediates platelet-monocyte adhesion. *J Clin Invest* **79**, 867-874.

Silverstein RL, Asch AS and Nachman RL (1989) Glycoprotein IV mediates thrombospondin-dependent platelet-monocyte and platelet-U937 cell adhesion. *J Clin Invest* **84**, 546-552.

Sims, P.J., Wiedmer, T., Esmon, C.T., Weiss, H.J. and Shattil, S.J. (1989) Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. *J.Biol.Chem.* **264**, 17049-17057.

Spangenberg P, Redlich H, Bergmann I, Lösche W, Götzrath M and Kehrel B (1993) The platelet glycoprotein IIb/IIIa complex is involved in the adhesion of activated platelets to leukocytes. *Thromb Haemost* **70**, 514-521.

Specker C, Perniok A, Brauckmann U, Siebler M and Schneider M (1998) Detection of cerebral microemboli in APS - introducing a novel investigation method and implications of analogies with carotid artery disease. *Lupus* **7**, S75-S80

Stenberg PE, McEver RP, Shuman MA, Jacques YV and Bainton DF (1985) A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J Cell Biol* **101**, 880-886.

Stenberg PE and Hill RJ (1998) Platelets and megakaryocytes. In: Lee GR, Foerster J, Lukens J, Paraskevas F, Greer JP and Rodgers GM, (Eds.) *Wintrobe's clinical hematology*, Tenth edn. pp. 615-660. Baltimore: Williams and Wilkins

Takaya, M., Ichikawa, Y., Kobayashi, N., Kawada, T., Shimizu, H., Uchiyama, M., Moriuchi, J., Watanabe, K. and Arimori, S. (1991) Serum thrombomodulin and anticardiolipin antibodies in patients with systemic lupus erythematosus. *Clin.Exp.Rheumatol.* **9**, 495-499.

Tannenbaum SH, Finko R and Cines DB (1986) Antibody and immune complexes induce tissue factor production by human endothelial cells. *J Immunol* **137**, 1532-1537.

Tans, G., Rosing, J., Thomassen, M.C., Heeb, M.J., Zwaal, R.F. and Griffin, J.H. (1991) Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles. *Blood* **77**, 2641-2648.

Thiagarajan, P. and Tait, J.F. (1990) Binding of annexin V/placental anticoagulant protein I to platelets. Evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. *J.Biol.Chem.* **265**, 17420-17423.

Tomer A (1997) A sensitive and specific functional flow cytometric assay for the diagnosis of heparin-induced thrombocytopaenia. *Br J Haematol* **98**, 648-656.

Triplett DA, Brandt JT, Musgrave KA and Orr CA (1988) The relationship between lupus anticoagulants and antibodies to phospholipid. *JAMA* **259**, 550-554.

Triplett DA (1993) Antiphospholipid antibodies and thrombosis. A consequence, coincidence, or cause? *Arch Pathol Lab Med* **117**, 78-88.

Triplett DA (1995) Protean clinical presentation of antiphospholipid-protein antibodies (APA). *Thromb Haemost* **74**, 329-337.

Tsutsumi A, Matsuura E, Ichikawa K, Fujisaku A, Mukai M, Kobayashi S and Koike T (1996) Antibodies to β_2-glycoprotein I and clinical manifestations in patients with systemic lupus erythematosus. *Arthritis Rheum* **39**, 1466-1474.

Tsutsumi A, Ichikawa, Y., Matsuura E and Koike T (1998) Anti-β2-glycoprotein I antibodies. *Lupus* **7**, S98-S102

Vaarala O, Puurunen M, Aho K, Manninen V and Palosuo T (1996) Antibodies to prothrombin imply a risk of myocardial infarction in middle-aged men. *Thromb Haemost* **75**, 456-459.

Vaarala O (1998) Antiphospholipid antibodies and myocardial infarction. *Lupus* **7**, S132-S134

Valant, P.A., Jy, W., Horstman, L.L., Mao, W.W. and Ahn, Y.S. (1998) Thrombotic thrombocytopenic purpura plasma enhances platelet-leucocyte interaction in vitro. *Br.J.Haematol.* **100**, 24-32.

Vazquez-Mellado, J., Llorente, L., Richaud-Patin, Y. and Alarcon-Segovia, D. (1994) Exposure of anionic phospholipids upon platelet activation permits binding of beta 2 glycoprotein I and through it that of IgG antiphospholipid antibodies. Studies in platelets from patients with antiphospholipid syndrome and normal subjects. *J.Autoimmun.* **7**, 335-348.

Vila L, de Castellarnau C, Borrell M, Fontcuberta J, Fabra A, Sanch MJ and Ruttlant ML (1985) Lupus anticoagulant and vascular prostacyclin production. *Thromb Haemost* **54**, 38 (Abstract)

Ware JA and Coller BS (1995) Platelet morphology, biochemistry, and function. In: Beutler E et al. (Eds.) *Williams Hematology*, 5th edn. pp. 1161-1201. New York: McGraw Hill

Wassermann A, Neisser A and Bruck C (1906) Eine serodiagnostiche Reaktion bei Syphilis. *Dtsch Med Wochenschr* **32**, 745-746.

Weiss HJ (1994) Scott syndrome: a disorder of platelet coagulant activity. *Semin Hematol* **31**, 312-319.

White JG (1994) Anatomy and structural organization of the platelet. In: Colman RW, Hirsh J, Marder VJ and Salzman EW, (Eds.) *Hemostasis and thrombosis: basic prinicples and clinical practice*, Third edn. pp. 397-413. Philadelphia: JB Lippincott Company

Wiener, H.M., Vardinon, N. and Yust, I. (1991) Platelet antibody binding and spontaneous aggregation in 21 lupus anticoagulant patients. *Vox Sang.* **61**, 111-121.

Wilkinson JM (1996) Monoclonal antibodies to platelet cell surface antigens. In: Watson SP and Authi KS, (Eds.) *Platelets: A practical approach*, pp. 155-171. Oxford: Oxford University Press

Wolf P (1967) The nature and significance of platelet products in human plasma. *Br J Haematol* **13**, 269-288.

Wong CS, Gamble JR, Skinner MP, Lucas CM, Berndt, M.C. and Vadas, M.A. (1991) Adhesion protein GMP140 inhibits superoxide anion release by human neutrophils. *Proc Natl Acad Sci USA* **88**, 2397-2401.

Wu G, Li F, Li P and Ruan C (1993) Detection of plasma alpha-granule membrane protein GMP-140 using radiolabeled monoclonal antibodies in thrombotic diseases. *Hemostasis* **23**, 121-128.

Wu K and Hoak JC (1974) A new method for the quantitative detection of platelet aggregates in patients with arterial insufficiency. *Lancet* **ii**, 926

Yamamoto N, Greco NJ, Barnard MR, Tanoue K, Yamazaki, M., Jamieson GA and Michelson AD (1991) Glycoprotein Ib (GPIb)-dependent and GPIb-independent pathways of thrombin-induced platelet activation. *Blood* **77**, 1740-1748.

Yamazaki, M., Asakura, H., Jokaji, H., Saito, M., Uotani, C., Kumabashiri, I., Morishita, E., Aoshima, K., Ikeda, T. and Matsuda, T. (1994) Plasma levels of lipoprotein(a) are elevated in patients with the antiphospholipid antibody syndrome. *Thromb.Haemost.* **71**, 424-427.

Zwaal RFA, Comfurius P and Bevers EM (1992) Platelet procoagulant activity and microvesicle formation. Its putative role in hemostasis and thrombosis. *Biochim Biophys Acta* **1180**, 1-8.

Zwaal RFA and Schroit AJ (1997) Pathophysiological implications of membrane phospholipid asymmetry in blood cells. *Blood* **89**, 1121-1132.

Appendix 1 Manufacturers cited in the thesis

Advanced Biotechnologies Ltd	Leatherhead, Surrey. KT22 7BE
Alpha Laboratories	Eastleigh, Hampshire. SO5 4NU
Beckman Coulter	Kingsmead Business Park, High Wycombe, Buckinghamshire. HP11 1JU
Becton-Dickinson UK Ltd	Cowley, Oxford. OX4 3LY
Bio Laboratories	Sheffield South, Yorkshire. S1 2DX
Boehringer Mannheim	Lewes, East Sussex. BN7 1LG
Brownes Ltd	Calcot, Reading. RG31 7SB
Chromogenix	Mölndal, Sweded.
Dade/Behring Diagnostics UK Ltd	Milton Keynes, Buckinghamshire. MK7 7AJ
Dako Ltd.	High Wycombe, Buckinghamshire. HP13 5RE
Diagnostic Reagents Ltd.	Thame, Oxon. OX9 3NY
Diagnostica Stago	Asnières, Cedex, France
Fisher Scientific	Loughborough, Leicestershire. LE11 5RG
FMC Bioproducts	Lichfield, Staffordshire. WS4 2EE
Genosys Biotechnologies	Cambridge, Cambridgeshire. CB2 4EF
Immuno Ltd.	Dunton Green, Sevenoaks, Kent. TN14 5HB
Life Technologies	Inchinnan Business Park, Paisley. PA5 9RF
Merck (BDH) Ltd.	Magna Park, Lutterworth, Leicestershire. LE17 4XN
Perkin Elmer Applied Biosystems	Warrington, Cheshire. WA3 7PB

Pharmacia	St Albans, Hertfordshire. AL1 3AW
Quadratech	Epsom, Surrey. KT17 2SB
R&D Systems Ltd.	Barton Lane, Abingdon, Oxon. OX14 3YS
Rho Reagents	Latchmoor Grove, Gerrards Cross, Buckinghamshire. SL9 8LN
Sarstedt Ltd.	Beaumont Leys, Leicestershire. LE4 1AW
Severn Biotech Ltd.	Kidderminster, Worcestershire. DY11 6TJ
Shield Diagnostics Ltd.	The Technology Park, Dundee. DD2 1SW
Sigma-Aldrich Chemical Co.	Poole, Dorset. NH17 7NH
Sysmex UK	Linford Wood, Milton Keynes, Buckinghamshire. MK14 6QF
Technoclone Ltd.	Dorking, Surrey. RH4 1EJ